Dkt. No. 96700/860

FLUORESCENT ASSAYS FOR PROTEIN KINASES

Priority Claim

[0001] This application claims the benefit of U.S. Provisional Application No. 60/439,359, filed January 10, 2003, and of U.S. Provisional Application No. 60/505,097, filed September 22, 2003, the contents of both of which are hereby incorporated by reference in their entirety into the subject application.

Statement of Government Support

[0002] The invention disclosed herein was made with U.S. Government support under grant number GM45989 from the National Institutes of Health, U.S. Department of Health and Human Services. Accordingly, the U.S. Government has certain rights in this invention.

Field of the Invention

[0003] The present invention relates to fluorescently-labeled peptide substrates for protein kinases and their uses in assays for protein kinases.

Background of the Invention

[0004] Throughout this application various publications are referred to in parenthesis or by reference number. Citations for these references may be found at the end of the specification immediately preceding the claims. The disclosures of these publications are hereby incorporated by reference in their entireties into the subject application to more fully describe the art to which the subject application pertains.

[0005] Cells recognize and respond to environmental stimuli via activation of intracellular biochemical pathways primarily comprised of protein kinases. These enzymes catalyze the phosphorylation of serine, threonine, and/or tyrosine residues on protein substrates. More specifically, protein kinases catalyze the transfer of the γ-phosphoryl group of adenosine triphosphate (ATP) to the hydroxyl moieties of serine, threonine, and tyrosine. This deceptively modest reaction serves as a cornerstone for the extraordinarily complex phenomenon known as signal transduction, the biochemical

process by which information is transmitted from the cell membrane to the cytoplasm and cell nucleus.[62] For example, the binding event between growth factor and its receptor on the cell surface is signaled to the nucleus via protein kinase-mediated pathways. In response to this signal, genes are transcribed and the cell prepares itself for division.[36] Mitosis is subsequently driven by a fine choreography of temporally- and spatially-regulated signaling pathways that ensure the myriad of biochemical processes required for replication occur in their proper chronological order. In short, signal transduction serves as a biochemical mechanism that drives an extraordinary array of biological phenomena. However, it would be simplistic to view signaling pathways as the molecular equivalent of the interstate highway system. The latter is fixed both in time and space. By contrast, kinase-mediated pathways not only evolved to rapidly form in response to some environmental stimulus, but their role in cellular homeostasis is dependent upon their rapid disassembly once the environmental signal has been acknowledged. Furthermore, the nature of the cellular response is dependent upon when and/or where a specific pathway is activated as well as by what other pathways may be simultaneously operating.

[0006] Protein kinases participate in the pathways that drive a variety of other important processes including apoptosis[37]. Members of this large enzyme family have been the objects of intense scientific scrutiny due to their role in disease onset and progression. The potential number of protein kinases encoded by the mammalian genome has been estimated to exceed 1,000.[63] The protein kinase C (PKC) family of enzymes has been implicated in a wide variety of processes, including control of gene expression,[72] mitotic progression,[26-30,78] angiogenesis, carcinogenesis, metastasis, and insulin action.[73] Cyclic guanosine monophosphate (cGMP)-dependent protein kinase (PKG) plays a key role in the signaling pathways responsible for memory and learning.[54,55] Protein kinases, and the signal transduction pathways in which they participate, are now recognized to be medicinally attractive targets of opportunity.[1-4,74-76] Inhibitors of the protein kinase family not only hold great promise as therapeutic agents, but are also of profound utility in the characterization of signaling pathways.[5] Consequently, there has been widespread interest in developing sensors of protein kinase activity, species that could furnish a visual readout of both where and when specific intracellular kinases are activated in response to a stimulus.

[0007] The substrate specificity of any given protein kinase is typically defined as the preferred amino acid sequence that envelops the serine, threonine, or tyrosine residue

phosphorylated by the enzyme (consensus recognition sequences).[53] In addition, protein kinases are typically divided into two families on the basis of their active site specificity: those that phosphorylate the aromatic phenol of tyrosine and those that catalyze the phosphorylation of the aliphatic alcohols of serine and threonine. PKC, PKG, and cyclic adenosine monophosphate (cAMP)-dependent protein kinase (PKA) share a strong sequence homology, and all three comprise what is commonly referred to as the "ACG" subfamily of protein kinases. Not surprising, these enzymes display overlapping sequence specificities with respect to both substrate and inhibitor peptides. However, their active site specificities are remarkably different.[48,56] For example, whereas PKA is unable to phosphorylate alcohol-bearing residues that possess an α-stereocenter corresponding to that present in D-amino acids, both PKC and PKG readily phosphorylate residues containing this configuration.[48,56,57] Furthermore, the differences in active site specificity between these otherwise closely related protein kinases are not just limited to stereochemical biases. For example, PKC phosphorylates *meta*- and *para*-substituted phenols, whereas PKA and PKG do not.[22] Protein microarrays have been used to investigate essentially all of the protein kinases encoded by the yeast genome.[77]

[0008] A variety of approaches have been described to assess protein kinase activity, including using phosphorylation-specific antibodies[6,65,66] and cytoplasmic sampling with capillary electrophoresis.[38] Ng et al. reported the detection of phosphorylated (activated) PKCα via fluorescence resonance energy transfer (FRET) using cyanine-labeled anti-phosphoPKCα and antiphosphoThr^{250} antibodies in fixed cells.[6] In this particular case, the activity of PKCα activity is not directly measured, but is inferred by detecting a functional state of the enzyme. Nagai et al. described the imaging of PKA activity in cells expressing a protein composed of two green fluorescent protein (GFP) variants tethered by a PKA phosphorylation site.[7] Phosphorylation of this protein generates a 23% decrease in FRET between the two GFPs. More recently, changes in FRET of 20-35% and 25-50% have been reported using genetically encoded reporters of protein tyrosine kinase[69] and PKA[68] activities, respectively.

[0009] Other studies have used peptide substrates that possess an appended fluorophore positioned near the site of phosphorylation[8-10,51,70,71] The phosphorylation-induced change in fluorescence intensity in these systems is modest (<20%)[8-10] and, as a consequence, the use of these substrates has often been limited to *in vitro* experiments with purified kinases. Nonetheless, peptide substrates possess a number of inherent

advantages, including ready synthetic availability, straightforward modification with the wide array of commercially available fluorophores, and the potential for complete temporal and spatial control over both when and where the substrate is phosphorylated.[11-17] Accordingly, there has been a need for fluorescently-labeled peptide substrates for protein kinases which undergo large changes in fluorescent intensity upon phosphorylation and which are suitable for *in vitro* and *in vivo* applications.

Summary of the Invention

[0010] The present application describes peptide-based reporters of protein kinase activity and uses thereof. The kinase peptide substrate comprises a fluorophore that is positioned on the same amino acid that undergoes phosphorylation. This allows the fluorophore to be placed within angstroms of the phosphorylatable moiety. Consequently, fluorophore-tagged peptides can be produced which display a phosphorylation-induced change in fluorescence that is an order of magnitude greater than previously described fluorophore-bearing peptide/protein substrates of protein kinases. These substrates serve as the basis of highly sensitive assays of protein kinases which have utility for high-throughput screening of chemical libraries for drug discovery, as well as for a variety of applications in fields such as enzymology, cell biology, structural biology and immunology. These substrates serve as effective fluorescent sensors of protein kinase activity in cell lysates and living cells, as well as for purified protein kinases.

[0011] The present invention provides a method for identifying a chemical compound that inhibits a protein kinase, which comprises separately contacting the protein kinase with both the chemical compound and a fluorescently-labeled substrate for the protein kinase, and with the fluorescently-labeled substrate, under conditions suitable for phosphorylation of the fluorescently-labeled substrate by the protein kinase, and measuring fluorescence intensity, a smaller change in fluorescence intensity in the presence of both the chemical compound and the fluorescently-labeled substrate than in the presence of the fluorescently-labeled substrate indicating that the chemical compound inhibits the protein kinase; wherein the fluorescently-labeled substrate comprises a peptide and at least one fluorophore, wherein a fluorophore is attached to a serine, a threonine, or a tyrosine on at least one terminal end of the peptide, and wherein phosphorylation of the substrate by the protein kinase occurs at the terminal serine, the terminal threonine, or the

terminal tyrosine to which the fluorophore is attached and produces at least a 20% change in fluorescence intensity.

[0012] The present invention also provides a method for screening a plurality of chemical compounds not known to inhibit a protein kinase to identify a compound that inhibits the protein kinase, which comprises:

(a) separately contacting the protein kinase with both the plurality of chemical compounds and a fluorescently-labeled substrate for the protein kinase, and with the fluorescently-labeled substrate, under conditions suitable for phosphorylation of the fluorescently-labeled substrate by the protein kinase;

(b) determining whether a change in fluorescence intensity is smaller in the presence of both the plurality of chemical compounds and the fluorescently-labeled substrate than in the presence of the fluorescently-labeled substrate; and if so

(c) separately determining for each compound included in the plurality of chemical compounds if the change in fluorescence intensity is smaller in the presence of both the compound and the fluorescently-labeled substrate than in the presence of the fluorescently-labeled substrate, a smaller change in fluorescence intensity indicating that the compound inhibits the protein kinase, so as to thereby identify any compound included in the plurality of chemical compounds that inhibits the protein kinase; wherein the fluorescently-labeled substrate comprises a peptide and at least one fluorophore, wherein a fluorophore is attached to a serine, a threonine, or a tyrosine on at least one terminal end of the peptide, and wherein phosphorylation of the substrate by the protein kinase occurs at the terminal serine, the terminal threonine, or the terminal tyrosine to which the fluorophore is attached and produces at least a 20% change in fluorescence intensity.

[0013] The present invention further provides a method for identifying a chemical compound that inhibits a protein kinase in a living cell, which comprises comparing the fluorescence intensity when a fluorescently-labeled substrate for the protein kinase is introduced into a cell which has not been contacted with the chemical compound, with the fluorescence intensity when the fluorescently-labeled substrate is introduced into a cell which has been contacted with the chemical compound, a smaller change in fluorescence intensity when the cell has been contacted with the chemical compound indicating that the compound inhibits the protein kinase in the living cell; wherein the fluorescently-labeled substrate comprises a peptide and at least one fluorophore, wherein a fluorophore is attached to a serine, a threonine, or a tyrosine on at least one terminal end of the peptide,

and wherein phosphorylation of the substrate by the protein kinase occurs at the terminal serine, the terminal threonine, or the terminal tyrosine to which the fluorophore is attached and produces at least a 20% change in fluorescence intensity.

[0014] The invention provides a method of making a composition which comprises identifying a chemical compound as a protein kinase inhibitor by any of the methods described herein and admixing the compound with a carrier.

[0015] The invention also provides a method for determining if a protein kinase is active in a living cell, which comprises either introducing a fluorescently-labeled substrate for the protein kinase into the cell or contacting a lysate from the cell with the fluorescently-labeled substrate, and measuring fluorescence intensity, a change in fluorescence intensity indicating that the substrate has been phosphorylated by the protein kinase and that the protein kinase is active in the living cell; wherein the fluorescently-labeled substrate comprises a peptide and at least one fluorophore, wherein a fluorophore is attached to a serine, a threonine, or a tyrosine on at least one terminal end of the peptide, and wherein phosphorylation of the substrate by the protein kinase occurs at the terminal serine, the terminal threonine, or the terminal tyrosine to which the fluorophore is attached and produces at least a 20% change in fluorescence intensity.

[0016] In addition, the invention provides a method for diagnosing a disease state that is correlated with a known change in activity of a protein kinase compared to the activity of the protein kinase in a normal state, which comprises comparing the activity of the protein kinase in the normal state with the activity of the protein kinase in a state that is being diagnosed, a change in activity corresponding to the known change indicating that the state that is being diagnosed is a disease state; wherein the activity of the protein kinase is measured using a fluorescently-labeled substrate that is phosphorylated by the protein kinase, wherein the fluorescently-labeled substrate comprises a peptide and at least one fluorophore, wherein a fluorophore is attached to a serine, a threonine, or a tyrosine on at least one terminal end of the peptide, and wherein phosphorylation of the substrate by the protein kinase occurs at the terminal serine, the terminal threonine, or the terminal tyrosine to which the fluorophore is attached and produces at least a 20% change in fluorescence intensity.

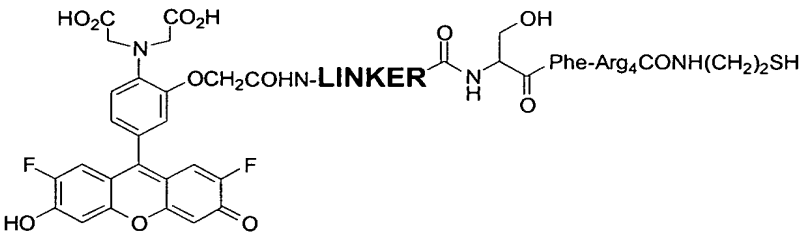
[0017] The invention also provides a substrate for a protein kinase, wherein the substrate comprises a peptide and at least one fluorophore, wherein a fluorophore is attached to a serine, a threonine, or a tyrosine on at least one terminal end of the peptide,

and wherein phosphorylation by the protein kinase of the terminal serine, the terminal threonine, or the terminal tyrosine to which the fluorophore is attached produces at least a 20% change in fluorescence intensity.

[0018] The invention further provides a substrate for a protein kinase, wherein the substrate comprises (1) a peptide comprising a serine, a threonine, or a tyrosine on a terminal end of the peptide; (2) at least one fluorophore, wherein a fluorophore is attached to the serine, the threonine, or the tyrosine on the terminal end of the peptide; and (3) a photolabile side chain attached to the serine, the threonine, or the tyrosine on the terminal end of the peptide, wherein the photolabile side chain blocks transfer of a phosphoryl group from adenosine triphosphate to a hydroxyl moiety of the serine, the threonine, or the tyrosine so that the substrate cannot be phosphorylated by a protein kinase until the photolabile side chain is removed from the substrate.

[0019] The invention provides a chemical compound comprising a peptide and a fluorophore, wherein the compound is selected from the group consisting of the compounds set forth in Table 3.

[0020] The invention also provides a chemical compound comprising a peptide and a fluorophore, wherein the compound has the structure:



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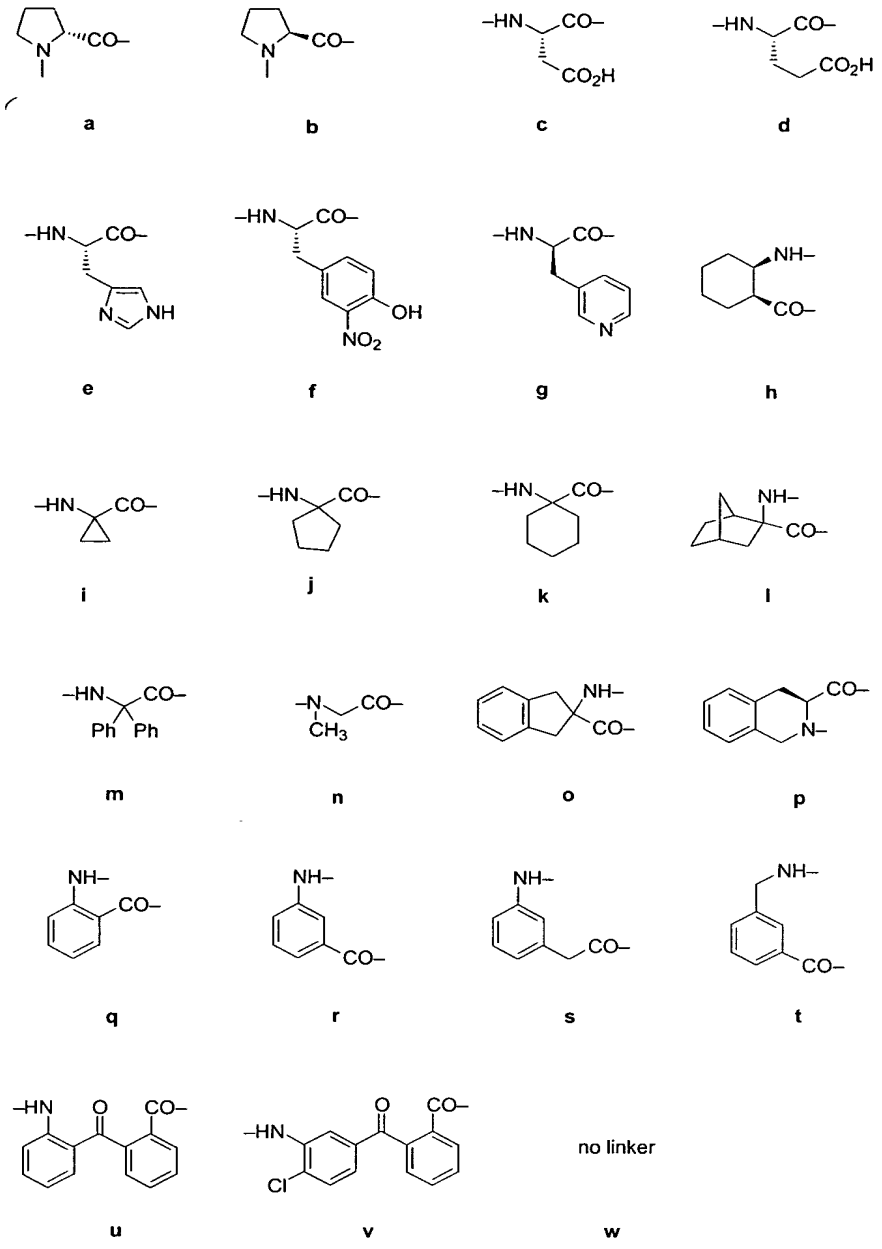
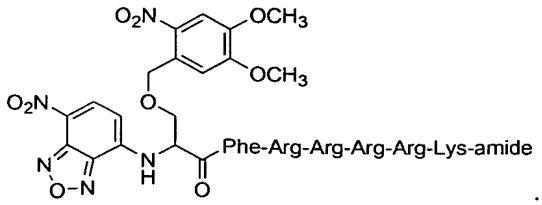
wherein the LINKER is selected from the group consisting of the following:



[0021] The invention also provides a chemical compound comprising a peptide and at least one fluorophore, wherein a fluorophore is attached to a serine, a threonine, or a tyrosine on at least one terminal end of the peptide.

[0022] The invention further provides a chemical compound having the structure: fluorophore-LINKER-X-FRRRRK-amide (SEQ ID NO:3); wherein F is phenylalanine; K is lysine; R is arginine; and X is serine, threonine, or tyrosine.

[0023] The invention provides a chemical compound having the structure



Brief Description of the Figures

[0024] Figure1A-1B. Excitation (A) and emission (B) spectra of the substrate peptide 2 (solid line) and the phosphorylated species 3 (dashed line). The emission spectra in B were produced by exciting the 7-nitrobenz-2-oxa-1,3-diazole (NBD) fluorophore at its λ_{max} (460 nm).

[0025] Figure 2. PKC activity in mitotic HeLa cell lysates. The PKC assay was initiated by addition of the lysate to the assay buffer. Fluorescence change as a function of incubation time in the presence of cell lysate (plot A), in the absence of cell lysate (plot B), in the presence of cell lysate and 4.5 μM staurosporine (plot C), and in the presence of cPKC immunodepleted cell lysate (plot D).

[0026] Figure 3. Immunodepletion of PKC from mitotic cell lysates. Western blot analysis performed with anti-PKCα antibody. Lane 1, crude cell lysate. Lane 2, cell lysate precleared with Protein-A Sepharose. Lane 3, cell lysate following immunodepletion of PKC. Lane 4, PKC immunoprecipitate following initial treatment of the mitotic cell lysate. Lane 5, PKC immunoprecipitate following a second treatment of the mitotic cell lysate.

[0027] Figure 4. TPA-induced time-dependent change in fluorescence intensity in HeLa cells containing the microinjected peptide 2 (plot A) and the microinjected peptide 2 in the presence of 20 μM GF 109203X (plot B). Time lapse fluorescence intensity measurements of TPA-stimulated HeLa cells microinjected with peptide 2. Combined data from 16 cells is furnished in plot A and from 14 cells is provided in plot B.

[0028] Figure 5. Irradiation of caged peptide (2) for various times intervals and the extent of conversion to uncaged sensor (1). See Example III for details.

[0029] Figure 6. Time-dependent change in fluorescence before and after *in situ* illumination of caged peptide. The caged peptide **2** (Example III) was incubated at 30 °C with PKCα and the change in fluorescence measured for 10 (A), 20 (B), or 30 (C) min.

Samples were then irradiated at the indicated time points. *Insert*: partial photolysis of **2** followed by a second exposure to brief illumination.

[0030] Figure 7. Fluorescence change as a function of irradiation time. Peptide **2** (Example III) was illuminated for (A) 0, (B) 15, (C) 30, (D) 45, and (E) 90 sec and PKCα-catalyzed activity subsequently sampled via fluorescence change.

[0031] Figure 8. Intracellular fluorescence change as a function of time following irradiation and/or TPA treatment. HeLa cells were microinjected with peptide **2** (Example III) and subsequently (A) irradiated and treated with TPA, (B) treated with TPA in the absence of light, and (C) irradiated in the absence of TPA.

Detailed Description of the Invention

[0032] The present invention provides a method for identifying a chemical compound that inhibits a protein kinase, which comprises separately contacting the protein kinase with both the chemical compound and a fluorescently-labeled substrate for the protein kinase, and with the fluorescently-labeled substrate, under conditions suitable for phosphorylation of the fluorescently-labeled substrate by the protein kinase, and measuring fluorescence intensity, a smaller change in fluorescence intensity in the presence of both the chemical compound and the fluorescently-labeled substrate than in the presence of the fluorescently-labeled substrate indicating that the chemical compound inhibits the protein kinase; wherein the fluorescently-labeled substrate comprises a peptide and at least one fluorophore, wherein a fluorophore is attached to a serine, a threonine, or a tyrosine on at least one terminal end of the peptide, and wherein phosphorylation of the substrate by the protein kinase occurs at the terminal serine, the terminal threonine, or the terminal tyrosine to which the fluorophore is attached and produces at least a 20% change in fluorescence intensity.

[0033] The invention also provides a method for screening a plurality of chemical compounds not known to inhibit a protein kinase to identify a compound that inhibits the protein kinase, which comprises:

(a) separately contacting the protein kinase with both the plurality of chemical compounds and a fluorescently-labeled substrate for the protein kinase, and with the fluorescently-labeled substrate, under conditions suitable for phosphorylation of the fluorescently-labeled substrate by the protein kinase;

(b) determining whether a change in fluorescence intensity is smaller in the presence of both the plurality of chemical compounds and the fluorescently-labeled substrate than in the presence of the fluorescently-labeled substrate; and if so

(c) separately determining for each compound included in the plurality of chemical compounds if the change in fluorescence intensity is smaller in the presence of both the compound and the fluorescently-labeled substrate than in the presence of the fluorescently-labeled substrate, a smaller change in fluorescence intensity indicating that the compound inhibits the protein kinase, so as to thereby identify any compound included in the plurality of chemical compounds that inhibits the protein kinase; wherein the fluorescently-labeled substrate comprises a peptide and at least one fluorophore, wherein a fluorophore is attached to a serine, a threonine, or a tyrosine on at least one terminal end of the peptide, and wherein phosphorylation of the substrate by the protein kinase occurs at the terminal serine, the terminal threonine, or the terminal tyrosine to which the fluorophore is attached and produces at least a 20% change in fluorescence intensity.

[0034] The invention further provides a method for identifying a chemical compound that inhibits a protein kinase in a living cell, which comprises comparing the fluorescence intensity when a fluorescently-labeled substrate for the protein kinase is introduced into a cell which has not been contacted with the chemical compound, with the fluorescence intensity when the fluorescently-labeled substrate is introduced into a cell which has been contacted with the chemical compound, a smaller change in fluorescence intensity when the cell has been contacted with the chemical compound indicating that the compound inhibits the protein kinase in the living cell; wherein the fluorescently-labeled substrate comprises a peptide and at least one fluorophore, wherein a fluorophore is attached to a serine, a threonine, or a tyrosine on at least one terminal end of the peptide, and wherein phosphorylation of the substrate by the protein kinase occurs at the terminal serine, the terminal threonine, or the terminal tyrosine to which the fluorophore is attached and produces at least a 20% change in fluorescence intensity.

[0035] The present application provides high throughput assays to identify protein kinase inhibitors. The efficacy of these inhibitors can be evaluated in cell based systems. Assays can be carried out using, for example, 96-well plates or chips which allow massive parallel measurements. As an analogous example, DNA microarrays have been used to sample time-dependent changes in gene expression patterns in response to environmental

stimuli.[60] Analogous methods are also being used to assess changes in protein levels and/or activities.[61,77]

[0036] The invention also provides a method for determining if a protein kinase is active in a living cell, which comprises either introducing a fluorescently-labeled substrate for the protein kinase into the cell or contacting a lysate from the cell with the fluorescently-labeled substrate, and measuring fluorescence intensity, a change in fluorescence intensity indicating that the substrate has been phosphorylated by the protein kinase and that the protein kinase is active in the living cell; wherein the fluorescently-labeled substrate comprises a peptide and at least one fluorophore, wherein a fluorophore is attached to a serine, a threonine, or a tyrosine on at least one terminal end of the peptide, and wherein phosphorylation of the substrate by the protein kinase occurs at the terminal serine, the terminal threonine, or the terminal tyrosine to which the fluorophore is attached and produces at least a 20% change in fluorescence intensity.

[0037] The assays and substrates described herein can be used as powerful diagnostic tools, where there is a change in protein kinase activity that is correlated with a disease state. For example, cAMP-dependent protein kinase has been implicated in the activation of the androgen receptor,[49] the upregulation of which is strongly correlated with cancer of the prostrate. Activation of the androgen receptor through signaling pathways that modulate phosphorylation may result in androgen-independent growth of prostate cancer cells.[49] In addition, overexpression of the epidermal growth factor receptor, as well as erbB-2, via gene activation is directly correlated with a poor clinical outcome in breast and ovarian cancer.[50]

[0038] The invention provides a method for diagnosing a disease state that is correlated with a known change in activity of a protein kinase compared to the activity of the protein kinase in a normal state, which comprises comparing the activity of the protein kinase in the normal state with the activity of the protein kinase in a state that is being diagnosed, a change in activity corresponding to the known change indicating that the state that is being diagnosed is a disease state; wherein the activity of the protein kinase is measured using a fluorescently-labeled substrate that is phosphorylated by the protein kinase, wherein the fluorescently-labeled substrate comprises a peptide and at least one fluorophore, wherein a fluorophore is attached to a serine, a threonine, or a tyrosine on at least one terminal end of the peptide, and wherein phosphorylation of the substrate by the protein kinase occurs at the terminal serine, the terminal threonine, or the terminal

tyrosine to which the fluorophore is attached and produces at least a 20% change in fluorescence intensity.

[0039] In any of the methods described herein, the change in fluorescence intensity when the substrate is phosphorylated by the protein kinase can be an increase in fluorescence intensity or a decrease in fluorescence intensity. In different embodiments, phosphorylation of the substrate by the protein kinase produces at least a 70% change in fluorescence intensity, or at least a 100% change in fluorescence intensity, or at least a 150% change in fluorescence intensity, or at least a 250% change in fluorescence intensity. In one embodiment, a metal ion chelator induces the change in fluorescence intensity. In different embodiments, the metal ion is a magnesium ion or a calcium ion.

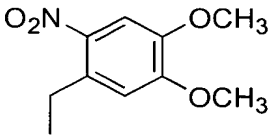
[0040] The methods described herein can be used with a protein kinase that is a purified protein kinase, a protein kinase that is obtained from a cell lysate, or a protein kinase in a living cell. Fluorescently-labeled substrates can be introduced into cells, for example, by microinjection of the substrates into the cells or by attaching the substrates to a substance that renders the substrate cell permeable. The cell can be a cancer cell. The cell can be a cell that is in a known phase of the cell cycle. The cell lysate can also be from a cancer cell. The cell lysate can be from a cell that is in a known phase of the cell cycle.

[0041] The issue of when protein kinases are activated in response to a stimulus has previously proven difficult to address. Lysis of synchronized cell populations followed by capture of the kinase in question and measurement of its activity furnishes some information concerning catalytic status. Intracellular fluorometric probes of protein kinase activity offer the potential of real time assessment of signaling activity under physiologically relevant conditions. The direct visualization of protein kinase activity in living cells provides a genuine assessment of the efficacy and selectivity of protein kinase inhibitors in a physiological setting. In addition, the ability to visualize the activity of a protein kinase in real time furnishes a direct measurement of the activation of specific signaling pathways in response to extracellular stimuli.

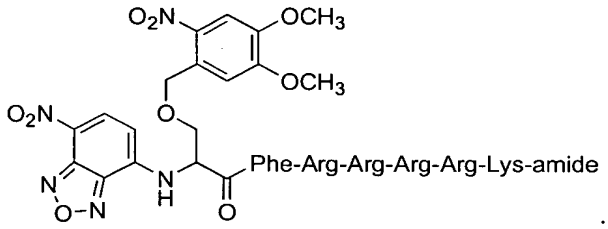
[0042] The assays described herein can be used with inert or "caged" substrates[11-14,58,59,64] that are quiescent until activated by light or other means. In such cases, the substrate cannot be phosphorylated by the protein kinase until the substrate is activated. Such substrates are especially useful for use with whole cell assays, where the substrate can be activated when the cell is in a desired state, for example in a desired phase of the

cell cycle. In addition, for example, for assays of PKA, photolytic release of cAMP from a membrane permeant ester, DMNB-cAMP, can be used.[67,68]

[0043] The caged substrate can comprise a caged serine, a caged threonine, or a caged tyrosine. The caged substrate can comprise a serine, a threonine, or a tyrosine with a photolabile side chain that blocks transfer of a phosphoryl group from adenosine triphosphate to a hydroxyl moiety of the serine, the threonine, or the tyrosine. The photolabile side chain can comprise the structure



A preferred caged substrate is



[0044] In any of the methods described herein, the substrate can be specific for a protein kinase subtype, for example protein kinase C, or isoforms α, β, and γ of protein kinase C. In other examples, the substrate is specific for protein kinase A, protein kinase B, protein kinase D, protein kinase G, Ca^{+}/calmodulin-dependent protein kinase, mitogen-activated protein kinase, protein kinase mos, protein kinase raf, protein tyrosine kinase, tyrosine kinase abl, tyrosine kinase src, tyrosine kinase yes, tyrosine kinase fps, tyrosine kinase met, cyclin-dependent protein kinase, or cdc2 kinase.

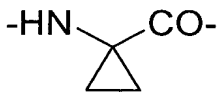
[0045] In one embodiment of the peptide substrate, one fluorophore is attached to one terminal end of the peptide. The terminal end can be the C-terminal end of the peptide or the N-terminal end of the peptide. In another embodiment, a fluorophore is attached to each terminal end of the peptide. In further embodiments, fluorophores with distinct photophysical properties are attached to different terminal ends of the peptide, or attached to one or both terminal ends and any nonterminal site on the peptide. For example, one fluorophore can be attached to a terminal end of the peptide and a second fluorophore,

with photophysical properties distinct from the first fluorophore, can be attached to any nonterminal site on the peptide.

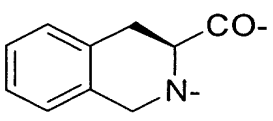
[0046] In cases where multiple fluorophores are attached to the substrate, the phosphorylation of the substrate by the protein kinase can also be detected using fluorescence resonance energy transfer (FRET) (e.g.[6,7,68,69]).

[0047] The fluorophore can be a 7-nitrobenz-2-oxa-1,3-diazole derivative or a fluorescein derivative. In other examples, the fluorophore can comprise a dansyl derivative, an acridine derivative, an Alexa Fluor derivative, a BODIPY derivative, an Oregon Green derivative, a Rhodamine Green derivative, a Rhodamine Red-X derivative, a Texas Red derivative, a Cascade Blue derivative, a Cascade Yellow derivative, a Marina Blue derivative, a Pacific Blue derivative, an AMCA-X derivative, or a coumarin derivative.

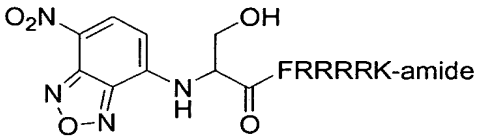
[0048] The fluorophore can be attached to the peptide by a linker. The linker can be a metal chelating linker. Preferably, the linker comprises a turn to position the fluorophore in a location closer to the terminal serine, the terminal threonine or the terminal tyrosine than the location the fluorophore would occupy in the absence of a turn in the linker. The linker can comprise one or more amino acids. In one embodiment, the linker does not comprise more than one amino acid. In one embodiment, the linker comprises non-amino acid residues and/or non-natural residues. In different embodiments, the linker can comprise, for example, a carboxamide linker, an aminobenzoic acid linker, a sulfonamide linker, a urea linker, a thiourea linker, an ester linker, a thioester linker, an alkylamine linker, an arylamine linker, an ether linker, or a thioether linker. The linker can be N-methyl glycine, L-proline, D-proline,



or



[0049] In different embodiments, the substrate is selected from the group consisting of:



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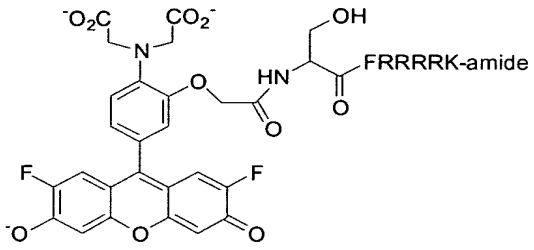

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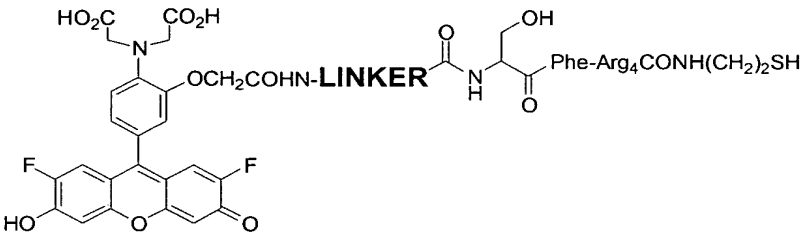
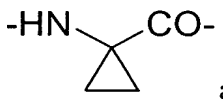


;

wherein F is phenylalanine, K is lysine, and R is arginine; and wherein the LINKER is selected from the group consisting of N-methyl glycine, L-proline, D-proline,



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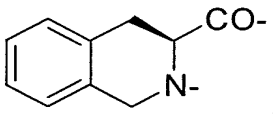

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[0050] The peptide substrate can further comprise any one or more of a lipid, a carbohydrate or a nucleic acid.

[0051] The protein kinase inhibitor, for example, can be a non-peptidyl compound or can comprise a peptide that is not phosphorylated by the protein kinase. The inhibitor can be a competitive inhibitor which binds to the active site of the protein kinase or a non-

competitive inhibitor which binds to a part of the protein kinase or the enzyme-substrate complex other than the active site.

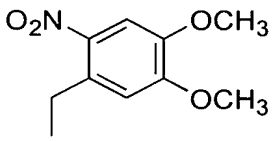
[0052] The invention provides a method of making a composition which comprises identifying a chemical compound as a protein kinase inhibitor by any of the methods described herein and admixing the compound with a carrier. The composition can be a pharmaceutical composition and the carrier a pharmaceutically acceptable carrier.

[0053] The invention provides a protein kinase inhibitor identified by any of the methods described herein, wherein the compound was not previously known to inhibit the protein kinase.

[0054] The invention provides a substrate for a protein kinase, wherein the substrate comprises a peptide and at least one fluorophore, wherein a fluorophore is attached to a serine, a threonine, or a tyrosine on at least one terminal end of the peptide, and wherein phosphorylation by the protein kinase of the terminal serine, the terminal threonine, or the terminal tyrosine to which the fluorophore is attached produces at least a 20% change in fluorescence intensity.

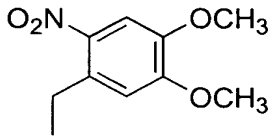
[0055] In one embodiment, a metal ion chelator induces the change in fluorescence intensity. In different embodiments, the metal ion is a magnesium ion or a calcium ion.

[0056] In one embodiment, the substrate cannot be phosphorylated by a protein kinase until the substrate is activated, for example until the substrate is activated by light. The substrate can comprise a serine, a threonine, or a tyrosine with a photolabile side chain that blocks transfer of a phosphoryl group from adenosine triphosphate to a hydroxyl moiety of the serine, the threonine, or the tyrosine. The photolabile side chain can comprise the structure

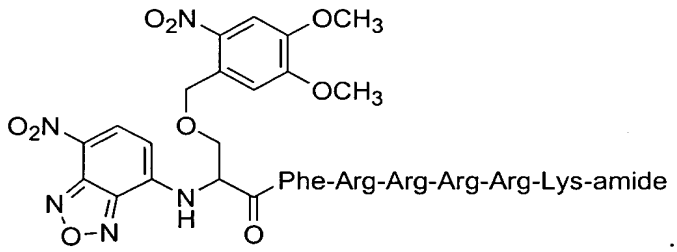


[0057] The invention also provides a substrate for a protein kinase, wherein the substrate comprises (1) a peptide comprising a serine, a threonine, or a tyrosine on a terminal end of the peptide; (2) at least one fluorophore, wherein a fluorophore is attached to the serine, the threonine, or the tyrosine on the terminal end of the peptide; and (3) a photolabile side chain attached to the serine, the threonine, or the tyrosine on the terminal end of the peptide, wherein the photolabile side chain blocks transfer of a phosphoryl

group from adenosine triphosphate to a hydroxyl moiety of the serine, the threonine, or the tyrosine so that the substrate cannot be phosphorylated by a protein kinase until the photolabile side chain is removed from the substrate. The photolabile side chain can comprise the structure



A preferred substrate with a photolabile side chain is



[0058] In one embodiment, after removal of the photolabile side chain from the substrate, phosphorylation by a protein kinase of the terminal serine, the terminal threonine, or the terminal tyrosine to which the fluorophore is attached produces at least a 20% change in fluorescence intensity. In one embodiment, a metal ion chelator induces the change in fluorescence intensity. In different embodiments, the metal ion is a magnesium ion or a calcium ion.

[0059] The change in fluorescence intensity when the substrate is phosphorylated by the protein kinase can be an increase in fluorescence intensity or a decrease in fluorescence intensity. In different embodiments, phosphorylation of the substrate by the protein kinase produces at least a 70% change in fluorescence intensity, or at least a 100% change in fluorescence intensity, or at least a 150% change in fluorescence intensity, or at least a 250% change in fluorescence intensity.

[0060] The substrate can be specific for a protein kinase subtype, for example protein kinase C, or isoforms α, β, and γ of protein kinase C. In other examples, the substrate is specific for protein kinase A, protein kinase B, protein kinase D, protein kinase G, Ca^{+}/calmodulin-dependent protein kinase, mitogen-activated protein kinase, protein kinase mos, protein kinase raf, protein tyrosine kinase, tyrosine kinase abl, tyrosine kinase

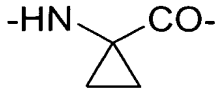
src, tyrosine kinase yes, tyrosine kinase fps, tyrosine kinase met, cyclin-dependent protein kinase, or cdc2 kinase.

[0061] The substrate can further comprise any one or more of a carbohydrate, a lipid or a nucleic acid.

[0062] In one embodiment, one fluorophore is attached to one terminal end of the peptide. The terminal end can be the C-terminal end of the peptide or the N-terminal end of the peptide. In another embodiment, a fluorophore is attached to each terminal end of the peptide. In further embodiments, fluorophores with distinct photophysical properties are attached to different terminal ends of the peptide or to any nonterminal site on the peptide. For example, one fluorophore can be attached to a terminal end of the peptide and a second fluorophore, with photophysical properties distinct from the first fluorophore, can be attached to any nonterminal site on the peptide.

[0063] In different embodiments, the fluorophore is a 7-nitrobenz-2-oxa-1,3-diazole derivative or a fluorescein derivative. The fluorophore can also be selected from the group consisting of, for example, but not limited to, a dansyl derivative, an acridine derivative, an Alexa Fluor derivative, a BODIPY derivative, an Oregon Green derivative, a Rhodamine Green derivative, a Rhodamine Red-X derivative, a Texas Red derivative, a Cascade Blue derivative, a Cascade Yellow derivative, a Marina Blue derivative, a Pacific Blue derivative, an AMCA-X derivative, and a coumarin derivative.

[0064] In different embodiments, the fluorophore is attached to the peptide by a linker. The linker can be a metal chelating linker. Preferably, the linker comprises a turn to position the fluorophore in a location closer to the terminal serine, the terminal threonine or the terminal tyrosine than the location the fluorophore would occupy in the absence of a turn in the linker. The linker can comprise one or more amino acids. In one embodiment, the linker does not comprise more than one amino acid. In one embodiment, the linker comprises non-amino acid residues and/or non-natural residues. The linker, for example, can be selected from the group consisting of a carboxamide linker, an aminobenzoic acid linker, a sulfonamide linker, a urea linker, a thiourea linker, an ester linker, a thioester linker, an alkylamine linker, an arylamine linker, an ether linker, and a thioether linker. In different embodiments, the linker is selected from the group consisting of N-methyl glycine, L-proline, D-proline,



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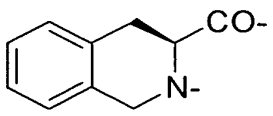

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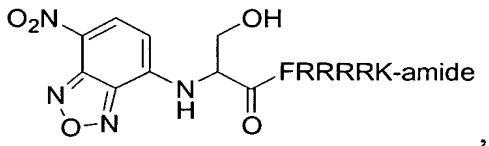
[0065] In different embodiments, the substrate is selected from the group consisting of:



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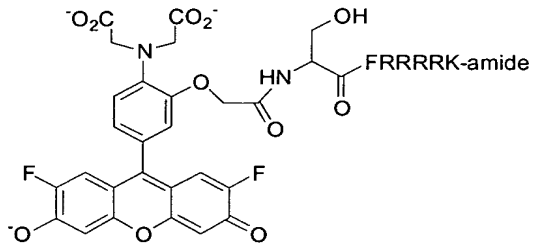

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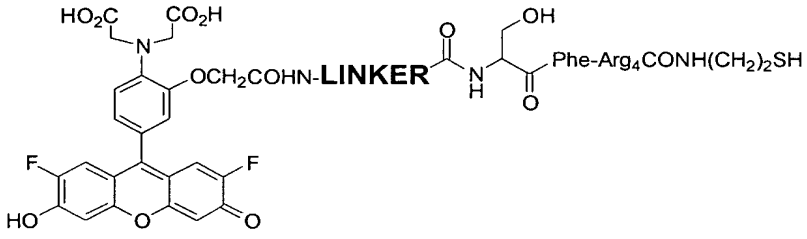
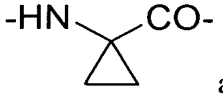


;

wherein F is phenylalanine, K is lysine, and R is arginine; and wherein the LINKER is selected from the group consisting of N-methyl glycine, L-proline, D-proline,



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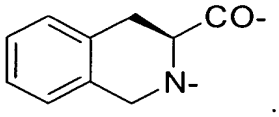
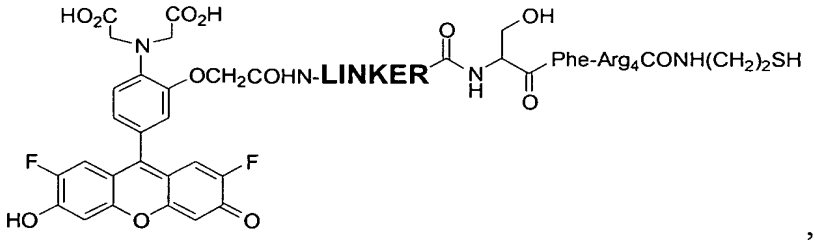


[0066] The invention provides a composition comprising any of the protein kinase substrates described herein and a carrier. In one embodiment, the composition is a pharmaceutical composition and the carrier is a pharmaceutically acceptable carrier.

[0067] The invention provides a chemical compound comprising a peptide and a fluorophore, wherein the compound is selected from the group consisting of the compounds set forth in Table 3. The invention provides a chemical compound selected from the group of compounds set forth in Table 3. The invention also provides a composition comprising any of the chemical compounds set forth in Table 3 and a carrier. In one embodiment, the composition is a pharmaceutical composition and the carrier is a pharmaceutically acceptable carrier.

[0068] The invention provides a chemical compound having the structure:



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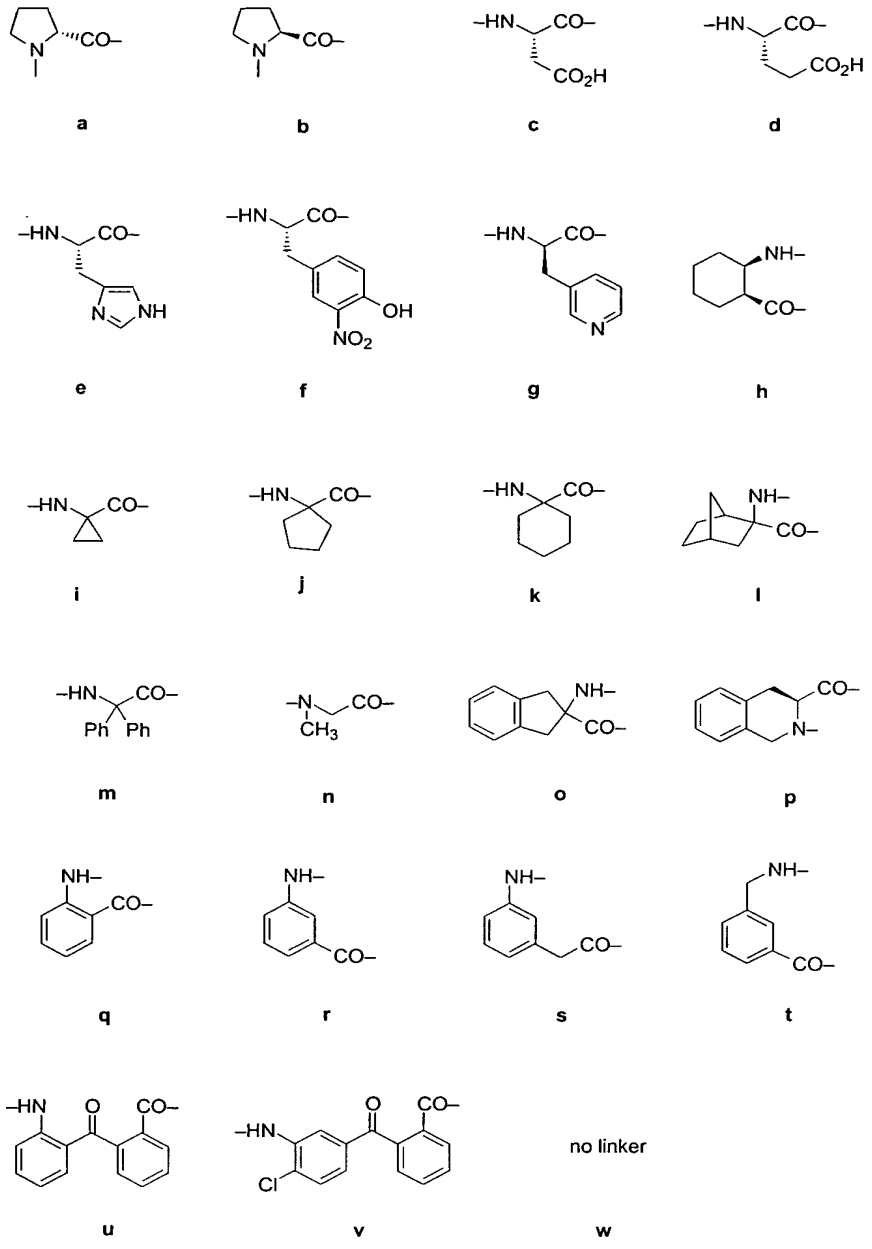
wherein the LINKER is selected from the group consisting of the following:

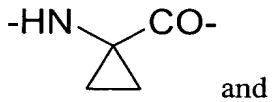


[0069] The invention provides a chemical compound having the structure: fluorophore-LINKER-X-FRRRRK-amide (SEQ ID NO:3); wherein F is phenylalanine; K is lysine; R is arginine; and X is serine, threonine, or tyrosine.

[0070] The fluorophore can be a 7-nitrobenz-2-oxa-1,3-diazole derivative or a fluorescein derivative. The fluorophore can be selected from the group consisting of, for example, but not limited to, a dansyl derivative, an acridine derivative, an Alexa Fluor derivative, a BODIPY derivative, an Oregon Green derivative, a Rhodamine Green derivative, a Rhodamine Red-X derivative, a Texas Red derivative, a Cascade Blue

derivative, a Cascade Yellow derivative, a Marina Blue derivative, a Pacific Blue derivative, an AMCA-X derivative, and a coumarin derivative.

[0071] The linker can be a metal chelating linker. Preferably, the linker comprises a turn to position the fluorophore in a location closer to the terminal serine, the terminal threonine or the terminal tyrosine than the location the fluorophore would occupy in the absence of a turn in the linker. The linker can comprise one or more amino acids. In one embodiment, the linker does not comprise more than one amino acid. In one embodiment, the linker comprises non-amino acid residues and/or non-natural residues. The linker can be selected from the group consisting of, for example, but not limited to, a carboxamide linker, an aminobenzoic acid linker, a sulfonamide linker, a urea linker, a thiourea linker, an ester linker, a thioester linker, an alkylamine linker, an arylamine linker, an ether linker, and a thioether linker. The linker can be selected from the group consisting of N-methyl glycine, L-proline, D-proline,



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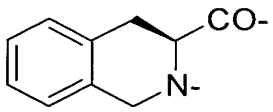
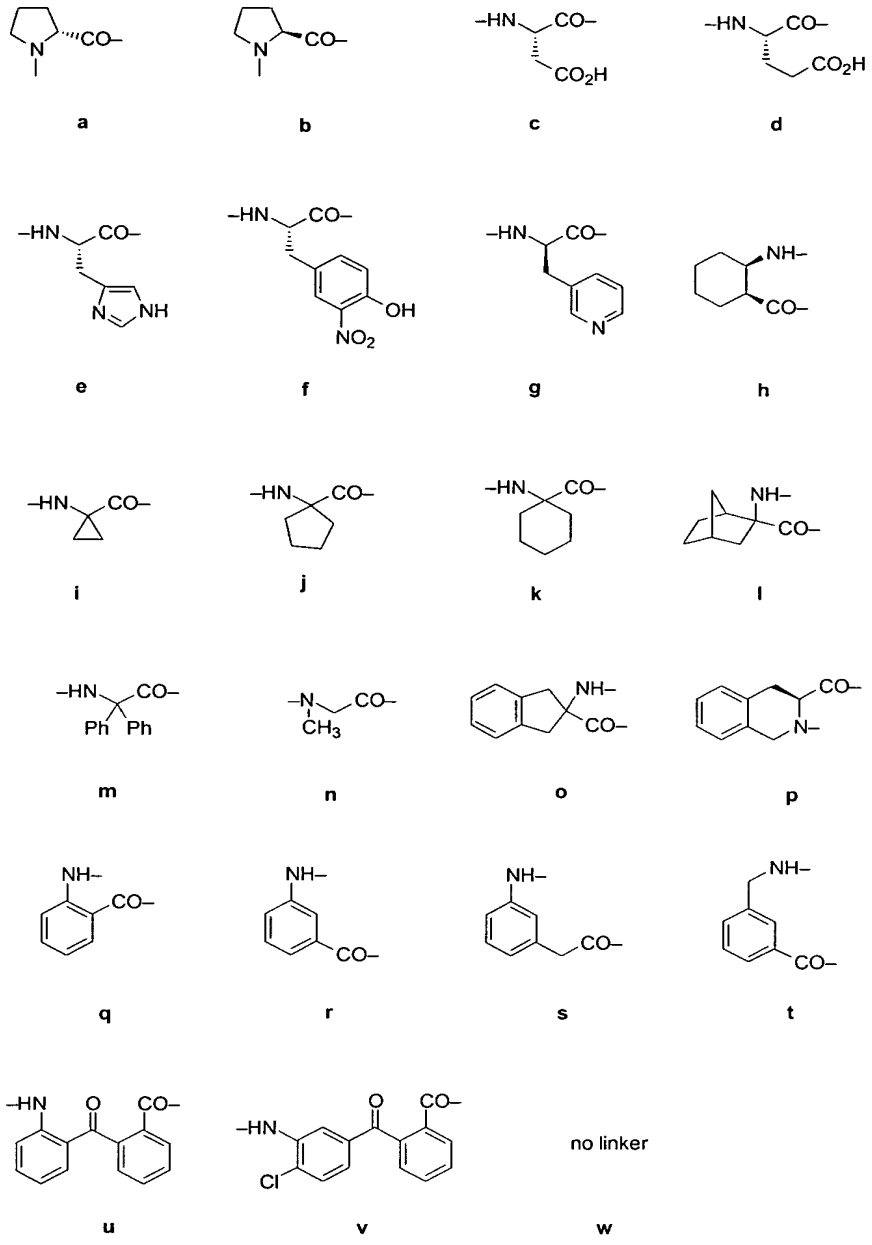


[0072] The LINKER can be selected from the group consisting of the following:



[0073] The chemical compound can act as a substrate for a protein kinase. The chemical compound can be specific for a protein kinase subtype, for example protein kinase C or isoforms α, β, and γ of protein kinase C. The chemical compound can be specific for, for example, but not limited to, protein kinase A, protein kinase B, protein kinase D, protein kinase G, Ca^{+}/calmodulin-dependent protein kinase, mitogen-activated protein kinase, protein kinase mos, protein kinase raf, protein tyrosine kinase, tyrosine

kinase abl, tyrosine kinase src, tyrosine kinase yes, tyrosine kinase fps, tyrosine kinase met, cyclin-dependent protein kinase, or cdc2 kinase.

[0074] The chemical compound can further comprise one or more of a carbohydrate, a lipid or a nucleic acid.

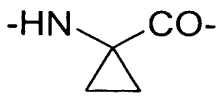
[0075] The invention also provides a chemical compound comprising a peptide and at least one fluorophore, wherein a fluorophore is attached to a serine, a threonine, or a tyrosine on at least one terminal end of the peptide.

[0076] The fluorophore can be attached to the C-terminal end of the peptide or to the N-terminal end of the peptide. A fluorophore can be attached to each terminal end of the peptide. Fluorophores with distinct photophysical properties can attached to different terminal ends of the peptide or to any nonterminal site on the peptide. For example, a first fluorophore can be attached to a terminal end of the peptide and a second fluorophore, with photophysical properties distinct from the first fluorophore, can be attached to any nonterminal site on the peptide. In different embodiments, the fluorophore is attached to serine, or to threonine, or to tyrosine on a terminal end of the peptide. If more than one fluorophore is used, the second fluorophore can be attached to serine, or to threonine, or to tyrosine on the second terminal end of the peptide. In further embodiments, the second fluorophore can be attached to any amino acid on a terminal end or on any nonterminal site on the peptide.

[0077] The fluorophore can be a 7-nitrobenz-2-oxa-1,3-diazole derivative or a fluorescein derivative. The fluorophore can be selected from the group consisting of, for example, but not limited to, a dansyl derivative, an acridine derivative, an Alexa Fluor derivative, a BODIPY derivative, an Oregon Green derivative, a Rhodamine Green derivative, a Rhodamine Red-X derivative, a Texas Red derivative, a Cascade Blue derivative, a Cascade Yellow derivative, a Marina Blue derivative, a Pacific Blue derivative, an AMCA-X derivative, and a coumarin derivative.

[0078] The fluorophore can be attached to the peptide by a linker. The linker can be a metal chelating linker. Preferably, the linker comprises a turn to position the fluorophore in a location closer to the terminal serine, the terminal threonine or the terminal tyrosine than the location the fluorophore would occupy in the absence of a turn in the linker. The linker can comprise one or more amino acids. In one embodiment, the linker does not comprise more than one amino acid. In one embodiment, the linker comprises non-amino acid residues and/or non-natural residues. The linker can be selected from the group

consisting of, for example, but not limited to, a carboxamide linker, an aminobenzoic acid linker, a sulfonamide linker, a urea linker, a thiourea linker, an ester linker, a thioester linker, an alkylamine linker, an arylamine linker, an ether linker, and a thioether linker. The linker can be selected from the group consisting of N-methyl glycine, L-proline, D-proline,



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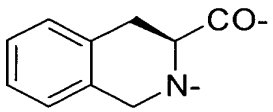
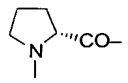


[0079] The linker can be selected from the group consisting of the following:



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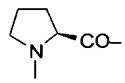

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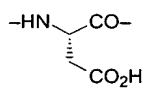

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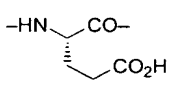

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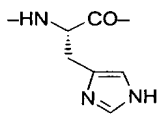

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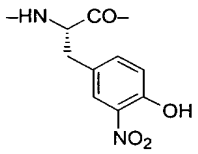

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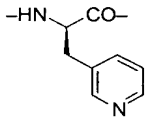

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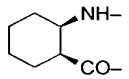

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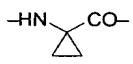

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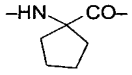

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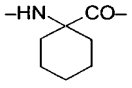

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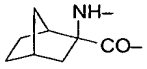

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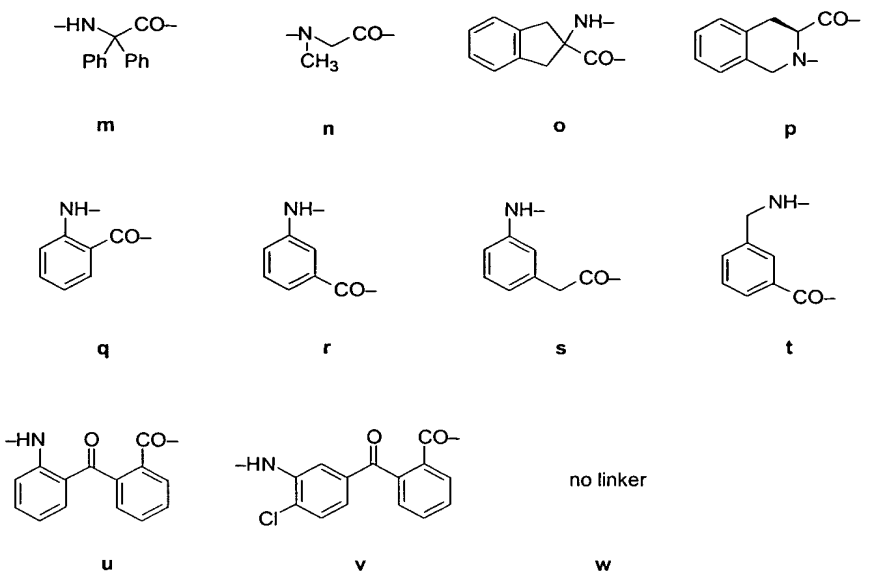
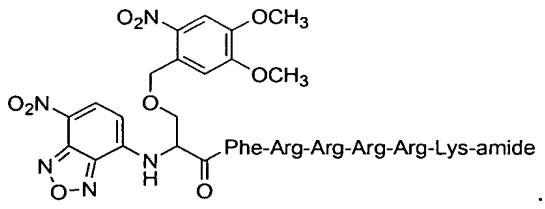


[0080] The chemical compound can act as a substrate for a protein kinase. The chemical compound can be specific for a protein kinase subtype, for example protein kinase C or isoforms α, β, and γ of protein kinase C. The chemical compound can be specific for, for example, but not limited to, protein kinase A, protein kinase B, protein kinase D, protein kinase G, Ca^{+}/calmodulin-dependent protein kinase, mitogen-activated protein kinase, protein kinase mos, protein kinase raf, protein tyrosine kinase, tyrosine kinase abl, tyrosine kinase src, tyrosine kinase yes, tyrosine kinase fps, tyrosine kinase met, cyclin-dependent protein kinase, or cdc2 kinase.

[0081] The chemical compound can further comprise any one or more of a carbohydrate, a lipid or a nucleic acid.

[0082] The invention provides a chemical compound having the structure



[0083] In one embodiment of chemical compounds described herein, a metal ion chelator induces a change in fluorescence intensity. In different embodiments, the metal

ion is a magnesium ion or a calcium ion. In one embodiment, the change in fluorescence intensity is at least a 20% change in fluorescence intensity.

[0084] The invention also provides a composition comprising any of the chemical compounds described herein and a carrier. In one embodiment, the composition is a pharmaceutical composition and the carrier is a pharmaceutically acceptable carrier. As used herein, the term "carrier" encompasses any of the standard pharmaceutical carriers, such as a sterile isotonic saline, phosphate buffered saline solution, water, and emulsions, such as an oil/water or water/oil emulsions.

[0085] The protein kinase substrates described herein can be used in assays to identify inhibitors of protein kinase.

[0086] The invention provides a method of treating an affliction in a subject, wherein the affliction is treated by inhibition of a protein kinase, where the method comprises administering to the subject a compound identified by any of the methods described herein for identifying a compound that inhibits a protein kinase, where the compound is administered to the subject in an amount effective to treat the affliction. The invention also provides for the use of a compound, identified by any of the methods described herein for identifying a compound that inhibits a protein kinase, for the preparation of a composition for treating an affliction in a subject, wherein the affliction is treated by inhibition of a protein kinase. The affliction can be a cancer, e.g.[89-92]

[0087] This invention will be better understood from the Experimental Details which follow. However, one skilled in the art will readily appreciate that the specific methods and results discussed are merely illustrative of the invention as described more fully in the claims which follow thereafter.

Experimental Details

EXAMPLE I

1. Materials and Methods

[0088] *General*-All chemicals were obtained from Aldrich, except for [γ-^{32}P] adenosine triphosphate (ATP) (obtained from New England Nuclear); bovine serum albumin, protease and phosphatase inhibitors (Sigma); protected amino acid derivatives and Rink resin (Advanced ChemTech); antibodies (Santa Cruz Biotech), and Liquiscint (National Diagnostics). The α-, ßII-, and γ-isoforms of protein kinase C were purchased from Panvera.

[0089] *Peptide and library synthesis*-Peptides and peptide libraries were synthesized using protocols analogous to those previously described.[24] Specific protocols are furnished for compounds **2** and **3**.

[0090] *Preparation of 7-nitrobenz-2-oxa-1,3-diazole (NBD)-NH-Ser-Phe-Arg$_4$-Lys-amide 2.* A standard 9-fluorenylmethoxycarbonyl(Fmoc)/benzotriazol-1-yloxy-tris(dimethylamino)phosphonium hexafluorophosphate (Bop) peptide synthesis protocol was employed to synthesize this peptide on the Rink resin via an automated peptide synthesizer. Each amino acid was attached according to the following program (for 2 g resin scale): (a) 3 x 30 ml CH_2Cl_2; (b) 1 x 20 ml of 30% piperidine in CH_2Cl_2 (1 min.); (c) 1 x 30 ml 30% piperidine in CH_2Cl_2 (20 min.); (d) 2 x 30 ml CH_2Cl_2; (e) 1 x 30 ml isopropanol; (f) 3 x 30 ml CH_2Cl_2; (g) 1 x 30 ml 4 equivalents of N-methyl morpholine in CH_2Cl_2; (h) 3 equivalents of Fmoc-protected amino acid, Bop, hydroxybenzotriazole and 6 equivalents of N-methyl morpholine in 30 ml CH_2Cl_2/DMF (1:1) (60-90 min.) (the coupling time was 90 min for all the Arg residues, and 60 min for the other residues); (i) 3 x 30 ml CH_2Cl_2; (j) 3 x 30 ml of 33% ethanol in CH_2Cl_2.; (k) 2 x 30 ml CH_2Cl_2. After completion of the desired amino acid sequence, the Fmoc group was removed with 30 ml of 30% piperidine (30 min.) and the resulting side chain-protected species, H_2N-Ser(tBu)-Phe-[Arg(Mtr)]$_4$-Lys(Boc)-[Rink Resin], was treated with 10 equivalents of NBD-Cl and N-methyl morpholine in 1:1 CH_2Cl_2/DMF (2 ml) for 24 hr, to furnish the resin-linked NBD-modified peptide: NBD-HN-Ser(tBu)-Phe-[Arg(Mtr)]$_4$-Lys(Boc)-[Rink Resin].

[0091] *Preparation of H_2N-Ser-Phe-Arg$_4$-Lys(NBD)-amide 3.* The protocol employed for peptide **2** was likewise used to synthesize **3** with the following exceptions: N-α-Fmoc-N-ε-1-(4,4-dimethyl-2,6-dioxocyclohex-1-ylidene)ethyl-Lys"Fmoc-Lys(Dde)" was used in place of Fmoc-Lys(tBoc). Upon synthesis of the Ac-HN-Ser(tBu)-Phe-[Arg(Mtr)]$_4$-

Lys(Dde)-[Rink Resin], the Dde protecting group was removed with hydrazine and the ε-amino moiety of Lys modified with NBD-Cl as described above. The NBD-peptide-Rink resins (i.e. **2** and **3**) were deprotected and cleaved as follows: each peptide-resin (100 mg) was individually transferred to a small reaction vessel containing 1 ml of a 9:1 trifluoroacetic acid/thioanisole mixture. The reaction vessel was then shaken at room temperature for 10 h. The resin was filtered, under reduced pressure, and then washed twice with trifluoroacetic acid. The filtrates were then combined (~5 ml) and an 8-10 fold volume of cold anhydrous ether (~ 50 ml) was added in a drop-wise fashion. The mixture was kept at 4 °C for at least 1 h. The precipitated peptide was then collected via filtration through a fine sintered glass filter funnel under a light vacuum. The precipitate was washed with cold anhydrous ether (2 x 5 ml), dissolved in 10 ml of water, and finally lyophilized to provide the crude peptide. The peptides were then purified on a preparative high performance liquid chromatography (HPLC) using three Waters radial compression modules (25 x 10 cm) connected in series (gradient A: 0.1% trifluoroacetic acid in water; solvent B: 0.1% trifluoroacetic acid in acetonitrile: 0-3 min. (100% A); a linear gradient from 3 - 20 min. (75% A and 25% B); a steep final linear gradient to 90% B for cleaning purposes). Peptides **2** and **3** were characterized by mass spectrometry.

[0092] In the case of the library of 415 compounds described herein (Table 3), peptide **1** was cleaved from the Rink resin with 90% trifluoroacetic acid/10% thioanisole, purified by HPLC, and then separately acylated with fluorescent carboxylic and sulfonic acids (**I**), condensed with aryl aldehydes (**II**), or directly arylated (**III**) via nucleophilic aromatic substitution. Carboxylic, sulfonic and related acid derivatives are as follows: fluorescamine, acridine-9-carboxylic acid, 5-dimethylamino-naphthalene-1-sulfonic acid, N,N'-diBoc-3-guanidino-naphthalene-2-carboxylic acid, 3-amino-naphthalene-2-carboxylic acid, 1-hydroxy-naphthalene-2-carboxylic acid, 3-di-*tert*-butoxycarbonylmethylamino-naphthalene-2-carboxylic acid, isoquinoline-3-carboxylic acid, quinoline-2-carboxylic acid, quinoline-8-carboxylic acid, 6-carboxytetramethylrhodamine succinimidyl ester, and 1-(3-(succinimidyloxycarbonyl)benzyl)-4-(5-(4-methoxyphenyl)oxazol-2-yl) pyridinium bromide. Aldehydes and related derivatives are as follows: naphthalene-2,3-dicarbaldehyde/cyanide, naphthalene-2,3-dicarbaldehyde/thiourea, naphthalene-2,3-dicarbaldehyde/dimethoxymethyl-amine, naphthalene-2,3-dicarbaldehyde/aminoguanidine, naphthalene-2,3-dicarbaldehyde/2-amino-pyridine, 2,4-

dinitro-benzaldehyde, 5-(4-dimethylamino-phenyl)-penta-2,4-dienal, bis-(2-hydroxy-phenyl)-methanone, bis-(4-hydroxy-phenyl)-methanone, 2-hydroxy-3,5-dinitro-benzaldehyde, anthracene-9-carbaldehyde, phenanthrene-9-carbaldehyde,pyrene-1-carbaldehyde, 4-dimethylamino-benzaldehyde, 2-hydroxy-4-diethylamine-benzaldehyde, 3-(4-dimethylamino-phenyl)-propenal, 4-methoxy-benzaldehyde, 2-hydroxyl-5-methoxy-benzaldehyde, 4-hydroxyl-benzaldehyde, 2,4-dimethoxybenzaldehyde, 2-hydroxy-naphthalene-1-carbaldehyde, 4-nitro-benzaldehyde, 2-nitrobenzaldehyde, 4-hydroxy-5-nitrobenzaldehyde, 2,6-dichloro-benzaldehyde, 4-styryl-benzaldehyde, and 3-phenyl-propenal. Aryl halides and related derivatives are as follows: NBD-Cl, 1-fluoro-2,4-dinitro-benzene, 6-chloro-5-nitro-quinoline, 2-chloro-5-trichloromethyl-nicotinonitrile, 2-bromo-pyrimidine, 2-bromo-4,6-bis-(4-chloro-phenyl)-pyrimidine, 4-chloro-3,5-dinitro-benzonitrile, 2-fluoro-5-nitro-benzoic acid, 5-fluoro-2,4-dinitro-phenylamine, 1-fluoro-4-nitro-2-trifluoromethyl-benzene, 4-chloro-2,8-bis-trifluoromethyl-quinoline, 4-chloro-2-phenyl-quinazoline, and 2-chloro-3,5-dinitro-pyridine.

[0093] *Radioactive PKC Assay*- Radioactive assays were performed in duplicate at 30 °C. The final assay volume totaled 40 µl and contained 62.5 mM HEPES (pH 7.4), 0.75 mM $CaCl_2$, 12.5 mM $MgCl_2$, 1 mM dithiothreitol, 0.5 mM EGTA, 7.5 µg/mL phosphatidylserine, 1.6 µg/mL diacylglycerol, and 10 ng of PKC. For the determination of the kinetic constants, the following concentrations were employed: 50 µM [γ-^{32}P]ATP (500-5000 cpm/pmol) and a substrate concentration that varied over a 10-fold range around the apparent K_m. Phosphorylation reactions were initiated by the addition of 10 µL of PKC from a stock solution (20 mM Tris-HCl at pH 7.5, 1 mM dithiothreitol, 1 mM EDTA, and 0.75 mg/mL bovine serum albumin) into 30 µL of assay buffer containing peptide substrate. Reactions were terminated after 5 min by spotting 25 µL aliquots onto phosphocellulose paper disks (2.1 cm in diameter). After 10 s, the disks were immersed in 10% glacial acetic acid and soaked with occasional stirring for at least 1 h. The acetic acid was decanted and the disks were collectively washed with four volumes of 0.5% H_3PO_4, 1 volume of water, followed by a final acetone rinse. The disks were air dried and then counted in a Beckman LS scintillation counter.

[0094] *Fluorescent PKC assay*-Fluorescence assays were performed in triplicate at 30 °C and initiated by addition of ATP to a 100 µL cuvette containing a 50 µL solution of PKC and peptide substrate **2**. Final conditions: 62.5 mM HEPES pH 7.4, 3 mM $MgCl_2$, 0.3 mM $CaCl_2$, 0.1 mM EGTA, 1 mM DTT, 0.5 µg/mL phosphatidylserine, 0.1 µg/mL

diacylglycerol, 1 mM ATP, and 13 nM PKC. After the addition of ATP, the solution was gently mixed and the time-dependent change in fluorescent intensity (excitation: 520 nm, emission: 560 nm) continuously monitored with a Photon Technology QM-1 spectrofluorimeter.

[0095] *Preparation of Mitotic Cell Extracts*-HeLa cells were synchronized for 16h in the presence of 250 ng/ml nocodazole and mitotic cells collected by selective detachment. Nocodazole was removed prior to lysis by washing the cells 2 times with 10 ml of ice-cold phosphate-buffered saline (1.5 mM KH_2PO_4, 8 mM $Na_2HPO_4 \cdot 7H_2O$, 2.6 mM KCl, 137 mM NaCl). The cells were then resuspended in a lysis buffer containing 50 mM Pipes pH 7.3, 5 mM $MgCl_2$, 0.2 mM EGTA, 1 M glycerol, 1 mM dithiothreitol, 0.5% Triton X-100, 1 mM PMSF, 10 μg/ml each of chymostatin, leupeptin and pepstatin and a 1:100 dilution of phosphatase inhibitor cocktail 1 (microcystin LR, cantharidin, and (-)-p-bromotetramisole) and phosphatase inhibitor cocktail 2 (sodium vanadate, sodium molybdate, sodium tartrate, and imidazole) (Sigma Chemical Co). The lysates were clarified by centrifugation at 4 °C for 5 minutes at 16,000g and the concentration of the supernatant determined using the Bio-Rad protein assay (Bio-Rad Laboratories). The supernatant was diluted with Dilution Buffer (50 mM Tris pH 7.5, 10 mM $MgCl_2$, 0.2 mM EGTA, 2 mM dithithreitol, 10 μg/ml each of chymostatin, leupeptin and pepstatin and phosphatase inhibitor cocktails 1 and 2 as described above) to a final concentration of 4 mg/ml.

[0096] *PKC activity in HeLa cell lysates*-The PKC assay was initiated via the addition of 10 μL of HeLa mitotic lysate to a cuvette containing a pre-incubated (30 °C) 110 μL assay solution containing (final concentration) 10 μM peptide **2**, 10 mM ATP, 750 μM $CaCl_2$, 12.5 mM $MgCl_2$, 500 μM EGTA, 1 mM dithithreitol, 7.5 μg/mL phosphatidylserine, 1.6 μg/mL diacylglycerol in 20 mM Tris at pH 7.5. The time-dependent change in fluorescent intensity (excitation: 520 nm, emission: 560 nm) was continuously monitored with a Photon Technology QM-1 spectrofluorimeter. The conventional PKCs α, β, and γ were immunodepleted from the HeLa cell lysate via (i) preclearance with protein A Sepharose, (ii) incubation with the monoclonal cPKC antibody PKC(MC5) (Santa Cruz Biotech), (iii) addition of protein A Sepharose and subsequent centrifugation, and (iv) repetition of steps iii and iv. Protein A Sepharose was used for mock immunodepleted lysates. The absence of PKCs α, β, and γ was confirmed

by Western blot analysis using antibodies targeted against the individual isoforms (Santa Cruz Biotech).

[0097] *Microinjection Studies*-Subconfluent, serum-starved (24 hr), HeLa cells were cultured on 22 mm coverslips in DMEM in a humidified atmosphere containing 5% CO_2. The NBD-containing peptide **2** (200 μM) in 50 mM Tris-HCl pH 7.2 was prefiltered through a 0.22 μm filter. Microinjections were performed with an Eppendorf (Brinkman Instruments, Westbury, NY) 5246 microinjection apparatus mounted on the microscope inside the environmental chamber. The peptide was estimated to be diluted 10-fold upon microinjection, using the equation for volume (V) flow through a capillary tube: $\frac{V}{t} = \frac{\pi pr^4}{8l\eta}$, where p is the difference in pressure at the ends of the tube (290 hPa), r the radius (0.05 μm) and l the length (10 μm) of the tube, η the viscosity of the injected solution (0.69 x 10^{-2} g/cm-sec), and t the total injection time (0.3 sec). The average volume of a fibroblast was taken as 2 pL.[18] 12-O-tetradecanoyl phorbol-13-acetate (TPA) (1 μM) was added to the media following microinjection to stimulate PKC activity. Time lapse images were collected with 2X2 binning using a Photometrics (Tuscon, Az) Sensys cooled CCD camera mounted on an Olympus IX 70 inverted microscope (Melville, NY) with a PlanApo 40X N. A. 0.75 objective, Ludl shutters (Hawthorne, NY), and a filter set with an excitation wavelength of 460 - 500 nm and an emission wavelength of 510 - 560 nm. Images were collected at 30 second intervals (300 msec exposure time). Fluorescence intensity measurements were corrected using values from a standard photobleaching curve generated from control experiments with microinjected HeLa cells that had not been treated with TPA. Analysis of cell intensities over time was conducted using I.P. Lab and Microsoft Excel (Redmond, OR). For experiments with the PKC inhibitor, the bisindolylmaleimide derivative GF 109203X (20 μM estimated intracellular concentration) was microinjected along with the NBD-peptide **2**.

2. Results and Discussion

[0098] The design of a fluorescent sensor of protein kinase activity requires a substrate that contains a fluorophore positioned either near the site of phosphorylation or at a more remote site that responds to phosphorylation via a conformational change. In addition to Ser, Thr, and Tyr residues, protein kinases will catalyze the phosphorylation of a wide variety of unnatural amino acid analogs in active site-directed peptides.[19-23] The

synthesis of these substrates is abetted by the fact that protein kinases phosphorylate alcohol-containing residues attached to the C- and/or N-terminus of appropriately designed peptides. Consequently, a wide variety of Ser analogs can be easily prepared and incorporated into the peptide substrate. For example, the peptide Ser-Phe-Arg-Arg-Arg-Arg-amide (SEQ ID NO:1) contains an N-terminal serine moiety that can be readily substituted with a virtually unlimited array of functional groups. Indeed, numerous N-substituted analogs of this peptide serve as are highly efficient substrates for the α, β, and γ isoforms of PKC.[24] It was thus reasoned that a peptide of the general structure fluorophore-Ser-Phe-Arg-Arg-Arg-Arg-amide (SEQ ID NO:1) would also function as an effective PKC substrate. The single compact fluorophore-serine residue contains a fluorescent reporter that is confined to within a few angstroms of the hydroxyl moiety, the site of imminent phosphorylation. Consequently, phosphorylation of the serine alcohol could exert a dramatic affect on the photophysical properties of the adjacent fluorophore.

[0099] The peptide H_2N-Ser-Phe-Arg-Arg-Arg-Arg-Lys-amide (**1**) (SEQ ID NO:2) was prepared using an Fmoc-based protocol, spatially segregated, and the free N-terminal amine subsequently modified with an array of fluorophore-containing carboxylic acids (**I**), aromatic aldehydes (**II**), and electron deficient aryl halides (**III**). The imines generated in **II** were also reduced to the corresponding secondary amines **IV** ($NaCNBH_3$) and transformed, via aromatic nucleophilic substitution, to a variety of tertiary amines **V** (Scheme 1). This spatially segregated fluorophore-substituted peptide-based library contains a total of 415 distinct chemical entities (Table 3) and was screened for changes in fluorescence intensity in the presence of PKC and ATP under activating conditions. Interestingly, the overwhelming majority (414) of these peptide-linked fluorophores displayed little (<10%) or no fluorescence change upon exposure to PKC. The obvious explanation is that phosphorylation of the peptide fails to induce the desired change in photophysical properties of the appended fluorophore. However, it is also possible that introduction of fluorophores, at various sites along the peptide framework, interferes with PKC-catalyzed phosphorylation. The latter possibility is unlikely, particularly for simple N-monosubstituted peptides, since PKC will phosphorylate peptides containing a wide variety of structurally diverse functionality attached at and/or near the phosphorylatable serine moiety.[24] Nevertheless, a few representative members of the library were evaluated in depth (Table 1). The 7-nitrobenz-2-oxa-1,3-diazole (NBD) derivative **2** displays the greatest change in fluorescence intensity. By contrast, the dansyl and acridine derivatives

exhibit phosphorylation-induced changes in fluorescence that are an order of magnitude less than that of NBD. The corresponding fluorescamine-treated peptide displays no fluorescence change as does the naphthalene-2,3-dicarbaldehyde/cyanide-treated species. One explanation for the poor fluorescence response of these particular peptides to PKC/ATP could be that these peptides serve as poor PKC substrates. This possibility was addressed by obtaining the PKCα-catalyzed K_m and V_{max} values using the [γ-^{32}P]ATP radioactive method.[25] As is clear from Table 1, all five peptides are reasonably effective substrates for PKCα.

[00100] The NBD-substituted peptide was subsequently examined in greater detail. The C-terminal-positioned Lys moiety allowed the comparison of fluorescence changes in response to phosphorylation as a function of NBD position (peptides **2** and **3**, Scheme 2) relative to the serine moiety. The efficacy of peptides **2** and **3** as PKC substrates were initially assessed using the radioactive ATP assay.[25] Both peptides serve as efficient substrates for pure recombinant PKC α, β, and γ with K_m and k_{cat} values similar to other PKC peptide substrates (Table 2).

[00101] The excitation and emission spectra of peptide **2** and its phosphorylated counterpart are furnished in Figure 1A-1B. The most dramatic difference in the excitation spectra of substrate and phosphorylated product is observed in the long wavelength region. Excitation of both NBD-peptide **2** and the corresponding phosphorylated derivative at 460 nm (λ_{max}) furnished a greater than 2-fold emission enhancement (>100%) in favor the phosphorylated peptide (Figure 1B). In addition, excitation at a longer wavelength (520 nm) produced an even larger (2.5-fold) relative enhancement in the emission intensity of the NBD fluorophore bound to the phosphorylated peptide. In contrast, no change in fluorescence intensity was detected following phosphorylation of peptide **3**. The k_{cat} and K_m values determined via spectrofluorimetry are modestly different from those acquired by the corresponding radioactive method (Table 2). These differences may reflect the slightly different conditions used in these assays, which were optimized to enhance fluorescence intensity changes (spectrofluorometric assay) or k_{cat}/K_m (radioactive assay). The variance between the kinetic constants generated by these two different assays is small and, using either assay, peptide **2** exhibits favorable properties as a PKC substrate for the α, β, and γ isoforms.

[00102] Several laboratories have reported the upregulation of PKC activity during mitosis.[26-29] PKC mediates the phosphorylation of the regulatory light chain of myosin-II

during mitosis.[30] Consequently, the ability of peptide **2** to report PKC activity in mitotic lysates from HeLa cells was examined. Crude mitotic cell extracts were prepared as previously described,[30] and the PKC assay initiated via addition of the cell lysate to an assay buffer containing peptide **2**. As shown in Figure 2, a linear increase in fluorescence intensity is observed in the first 10 minutes following addition of the cell lysate, which plateaus at approximately 1.7-fold above background. The protein kinase inhibitor staurosporine blocks the increase in fluorescence intensity observed upon mitotic cell lysate addition. Staurosporine,[31] like many protein kinase inhibitors,[32-33] targets a variety of protein kinases. Consequently, from these experiments, it is not clear whether the staurosporine-induced block of fluorescence is due to inhibition of PKC activity or the inhibition of other protein kinases that also catalyze the phosphorylation of peptide **2**.

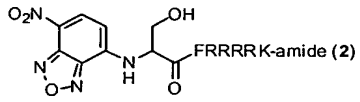
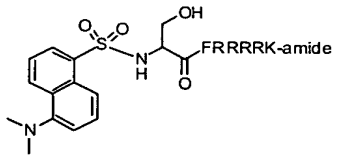
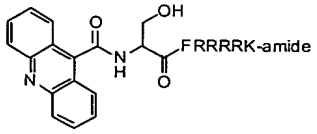
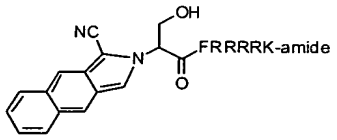
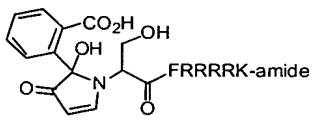
[00103] The question whether the peptide substrate **2** is selective for the conventional PKCs was addressed by immunodepleting the mitotic cell lysates of PKCs α, β, and γ using a commercially available antibody that recognizes all three PKC isoforms. Depletion of the individual α, β, and γ isoforms from the cell lysate was confirmed by immunoblot analysis using isoform-specific antibodies (shown for PKC α in Figure 3). Analogous Western blots were performed for the β and γ PKC isoforms as well (data not shown). The immunodepleted lysate (Figure 3, lane 3) was examined for PKC activity in the fluorescence assay. No change in fluorescence intensity was observed over the course of 1 hr (Figure 2, plot D). The latter observation is consistent with the notion that the fluorescence enhancement observed with crude mitotic lysates is due to PKC. It is unlikely that a protein kinase downstream from PKC is responsible for the observed enhancement in fluorescence, since this putative downstream kinase would have already been activated in the cell lysate prior to immunodepeletion of the conventional PKCs.

[00104] Given the enzymological and photophysical behavior displayed by peptide **2**, the *in vivo* visualization of PKC activity was examined in live cells. Serum-starved HeLa cells were microinjected with the NBD-modified peptide and the cells subsequently exposed to TPA, a tumor promoting phorbol ester that potently and specifically activates PKC.[34] A change in fluorescence intensity is evident within 4 minutes of TPA exposure and significantly so by 8 minutes. A comparison of the curves generated in the lysate (Figure 2) and live cell (Figure 4) assays demonstrate that both curves display an essentially linear increase in fluorescence intensity within the first 10 min of exposure to activated PKC, followed by a plateau phase shortly thereafter. The overall enhancement in

fluorescence intensity displayed by peptide **2** in living cells is 2-fold, whereas the cell lysate-based experiments furnish an overall 1.7-fold increase in fluorescence. In an additional series of experiments, the NBD-peptide PKC substrate was co-injected with the known PKC inhibitor, GF 109203X.[35] This inhibitor effectively blocks the TPA-induced enhancement in cellular fluorescence. Although the K_i value for GF 109203X is in the low nanomolar range, micromolar concentrations of GF 109203X were used to block *in vivo* PKC activity due to the presence of high intracellular levels of ATP.

[00105] In summary, a fluorescent substrate for PKC has been constructed using a strategy that positions the reporter-group directly on the residue undergoing phosphorylation. A library of fluorescently-labeled PKC peptide substrates was prepared. The lead derivative displays a phosphorylation-induced fluorescence change that allows the visualization of real time PKC activity in both cell lysates and living cells. Furthermore, immunodepletion experiments suggest that the fluorescently-tagged peptide is selectively, if not exclusively, phosphorylated by the conventional PKCs. The PKC biosensor strategy outlined herein takes advantage of the ease with which peptides can be modified to create libraries of structurally altered analogs. However, the inherent synthetic mutability of peptides is not just limited to library construction. For example, it may be possible to simultaneously monitor more than one protein kinase by affixing fluorophores with distinct photophysical properties to the N- or C-termini of appropriately designed active site-directed peptides. Furthermore, there exists the potential for temporal and spatial control over when and where the substrate is phosphorylated via the preparation of "caged" analogs[11-14] (see Example III). In addition to protein kinase C,[45] many other protein kinases are known to phosphorylate peptides that contain N- or C- terminally appended serine, threonine, or tyrosine residues. These protein kinases include cdc2 kinase,[47] cAMP-dependent protein kinase,[45] cyclic guanosine monophosphate (cGMP)-dependent protein kinase,[48] tyrosine kinase src,[19] and tyrosine kinase abl.[46] Consequently, the approaches demonstrated herein with respect to protein kinase C are expected to have broad applicability to protein kinases.

Table 1. PKCα-catalyzed phosphorylation of selected library members. Fluorescence enhancement upon phosphorylation (% Change) was obtained by spectrofluorimetry and K_m and k_{cat} values were acquired using the standard [γ-^{32}P]ATP radioactive assay.[25]

Fluorophore-peptide	Fluorophore Reagent	% Change	K_m (μM)	k_{cat} (min^{-1})
	NBD-Cl	150%	9.0 ± 1.0	380 ± 20
	Dansyl chloride	20%	28 ± 3	170 ± 10
	9-Acridinecarboxylic acid	20%	13 ± 2	20 ± 2
	Cyanide/Naphthalene -2,3-dicarbaldehyde	-	70 ± 1	220 ± 10
	Fluorescamine	-	280 ± 60	25 ± 4

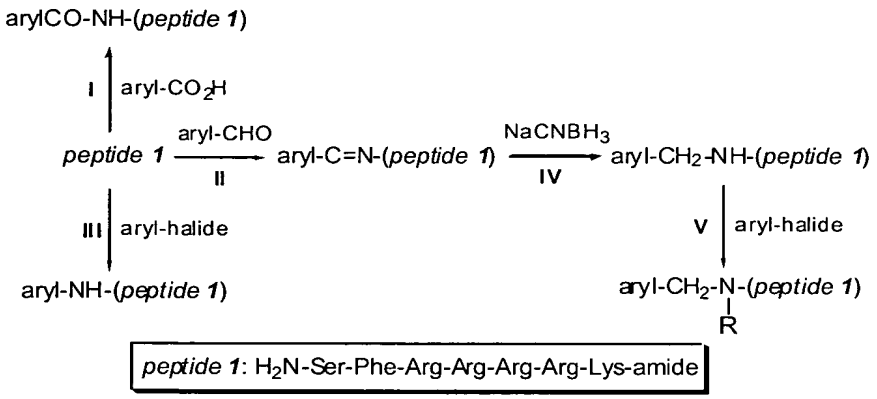
Abbreviations of amino acids: F = Phe = phenylalanine; K = Lys = lysine; R = Arg = arginine.

Table 2. Kinetic constants associated with the phosphorylation of peptides 2 and 3 by recombinant human PKCs α, β, and γ.

Substrate	Assay	Kinetic constants	PKCα	PKCβ	PKCγ
NBD-SFR_4K (peptide 2)	Radioactive	K_m (μM)	9.0 ± 1.0	9.2 ± 0.4	5.0 ± 1.0
		k_{cat} (min^{-1})	380 ± 20	180 ± 10	210 ± 20
		k_{cat}/K_m (min^{-1} μM^{-1})	42 ± 5	23 ± 2	42 ± 9
	Fluorescence	K_m (μM)	29 ± 3	27 ± 4	30 ± 5
		k_{cat} (min^{-1})	170 ± 30	94 ± 9	190 ± 40
		k_{cat}/K_m (min^{-1} μM^{-1})	5.9 ± 1	3.5 ± 0.6	6.3 ± 1.7
SFR_4K(ε-NBD) (peptide 3)	Radioactive	K_m (μM)	19 ± 1	Not determined	Not determined
		k_{cat} (min^{-1})	210 ± 10		
		k_{cat}/K_m (min^{-1} μM^{-1})	11 ± 1		
	Fluorescence	K_m (μM)	No Fluorescence Intensity Change		
		k_{cat} (min^{-1})			
		k_{cat}/K_m (min^{-1} μM^{-1})			

Abbreviations of amino acids: F = Phe = phenylalanine; K = Lys = lysine; R = Arg = arginine; R_4 = R-R-R-R; S = Ser = serine.

Sch me 1



Scheme 2

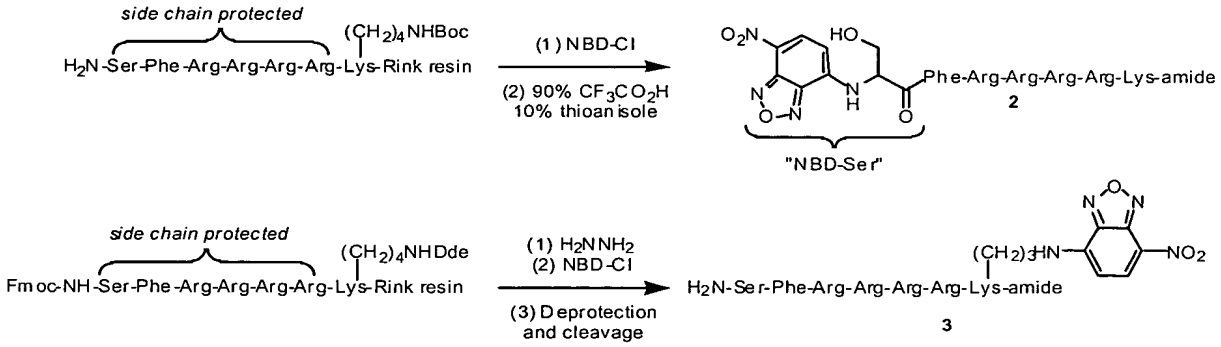
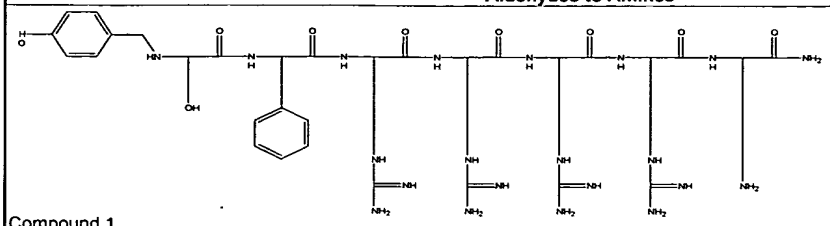
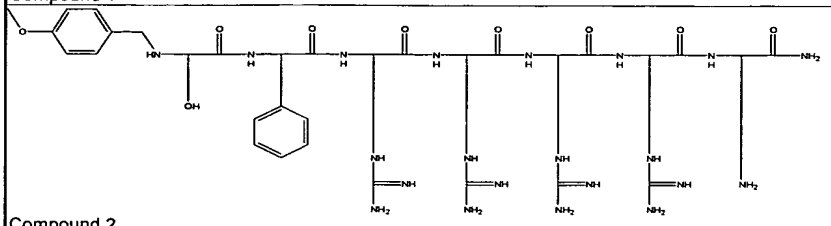
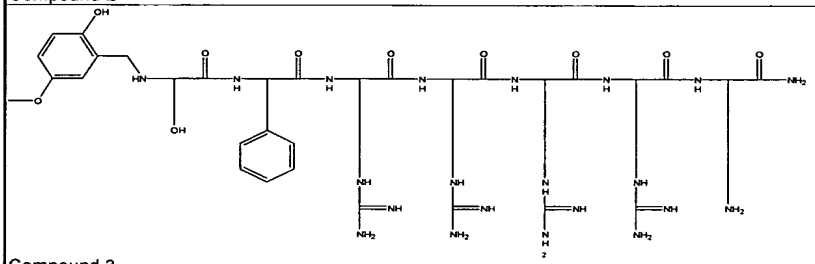
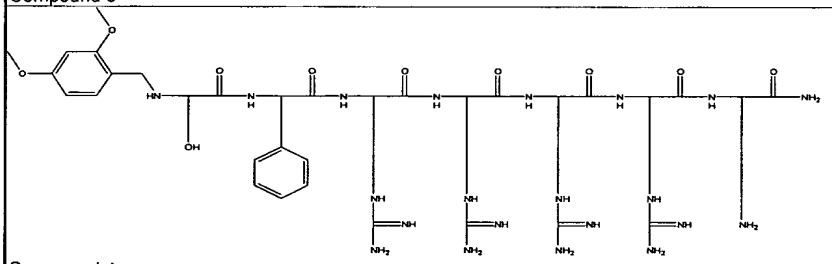
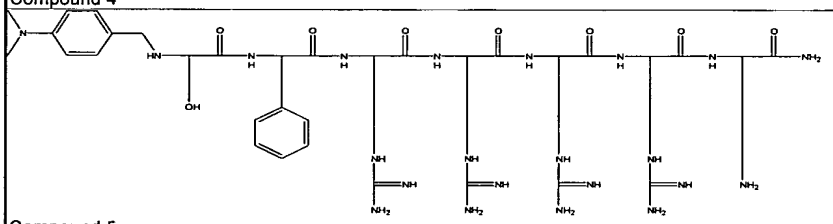
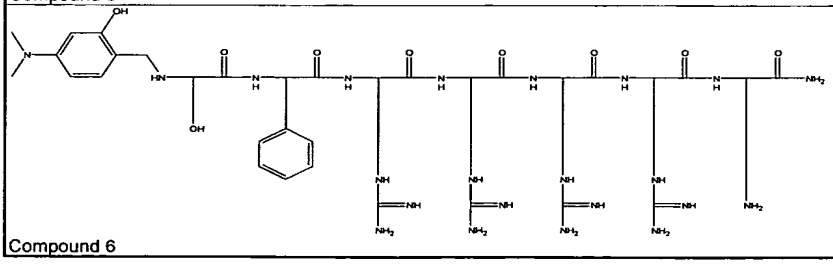
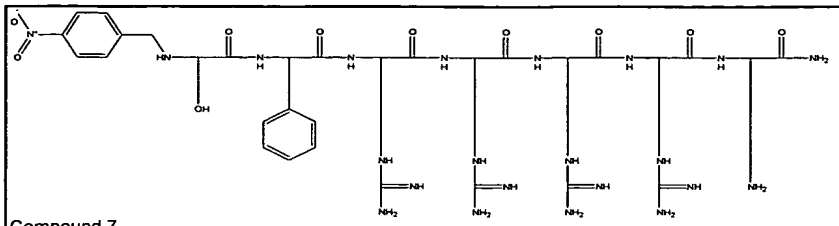
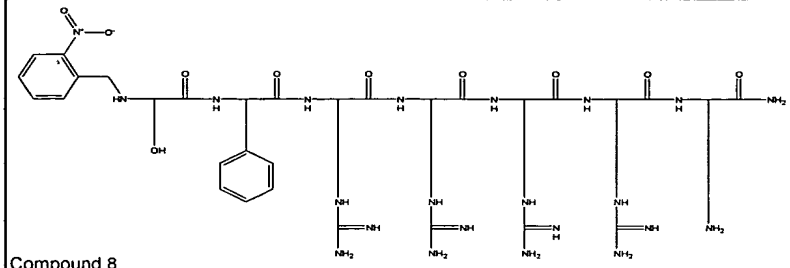
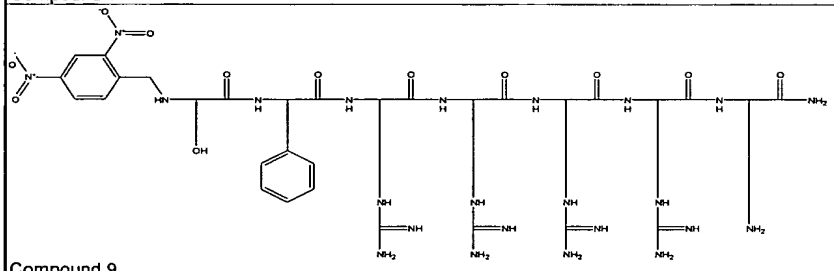
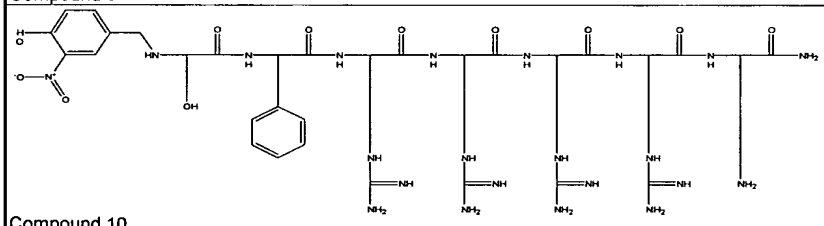
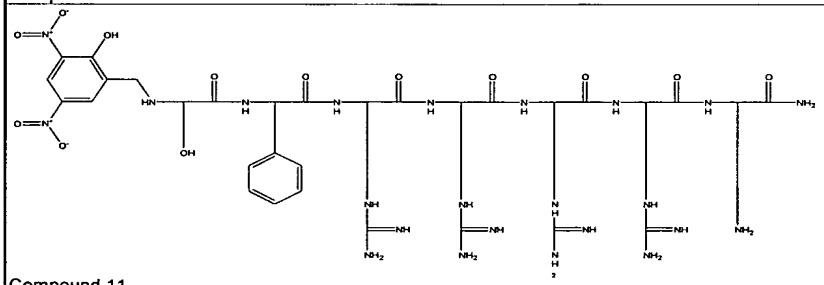
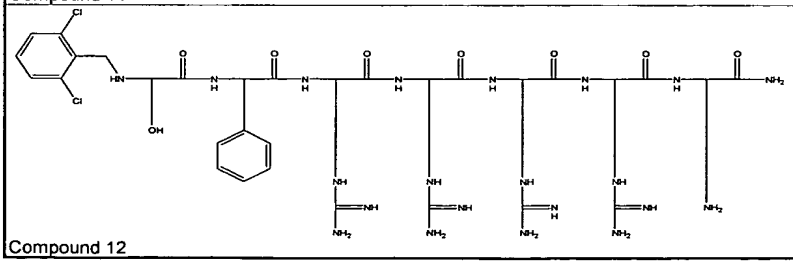
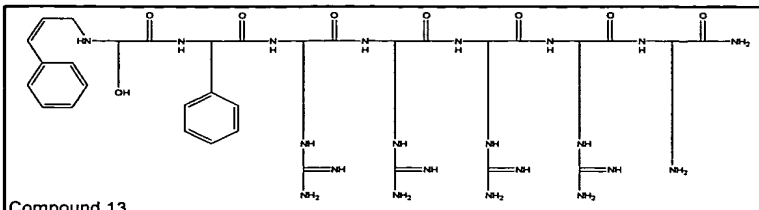
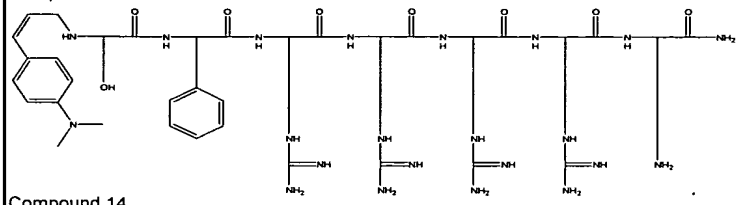
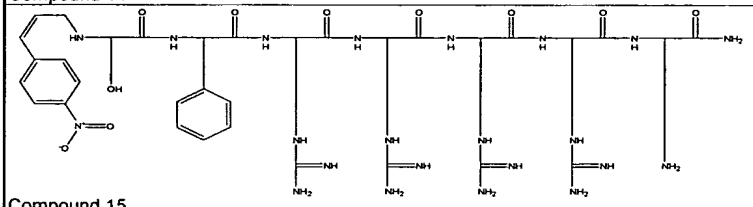
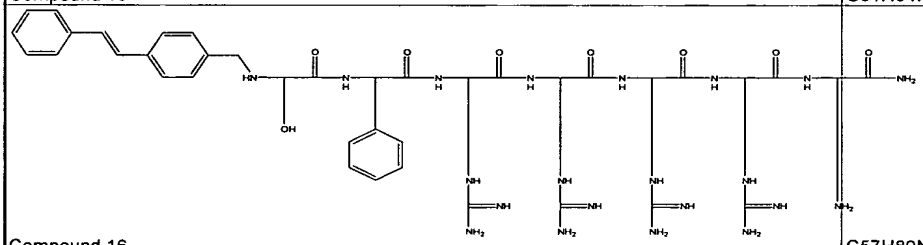
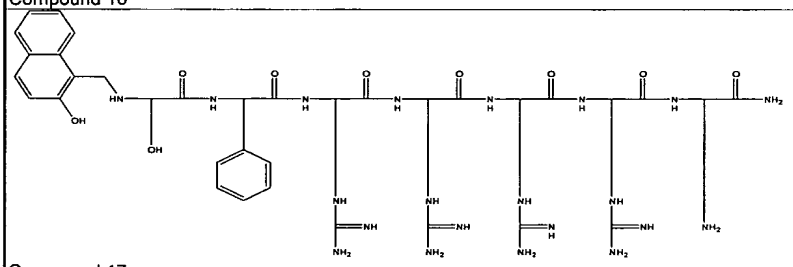
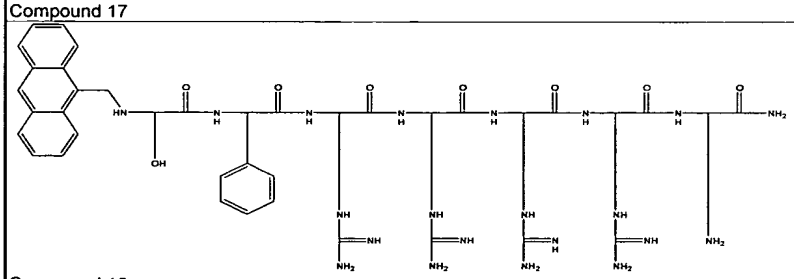
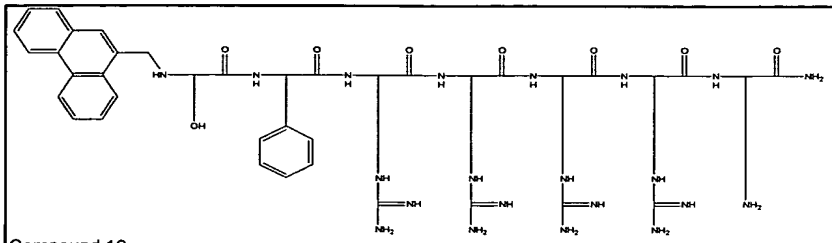
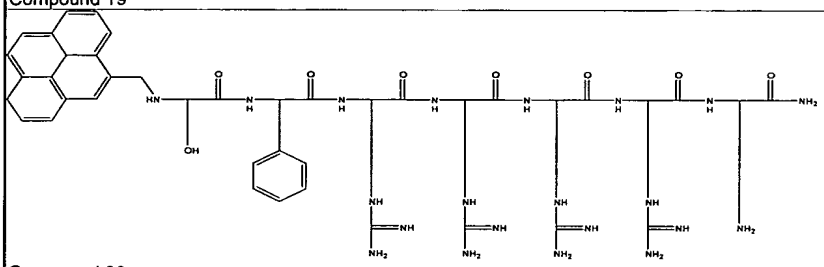
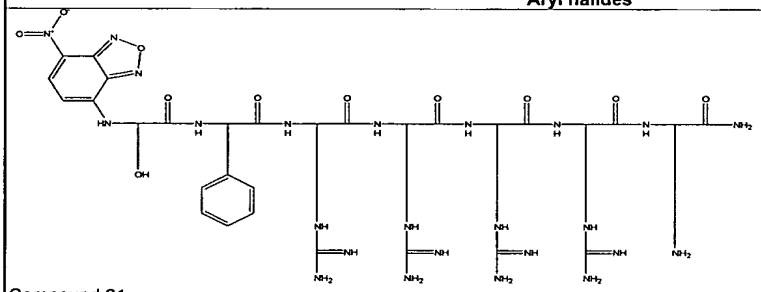
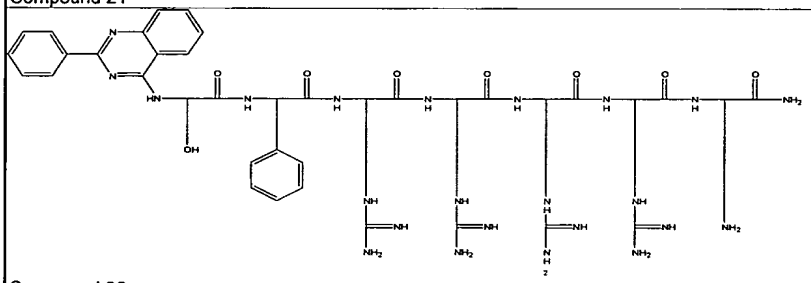
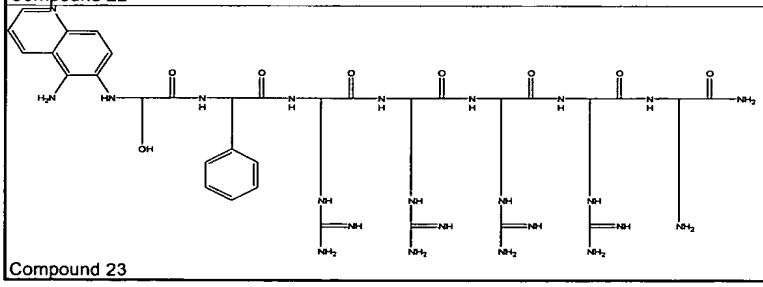


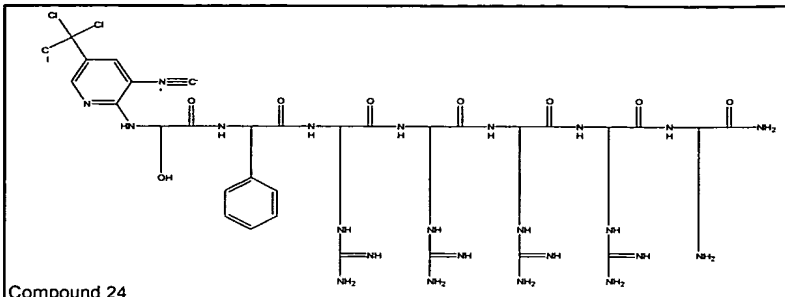
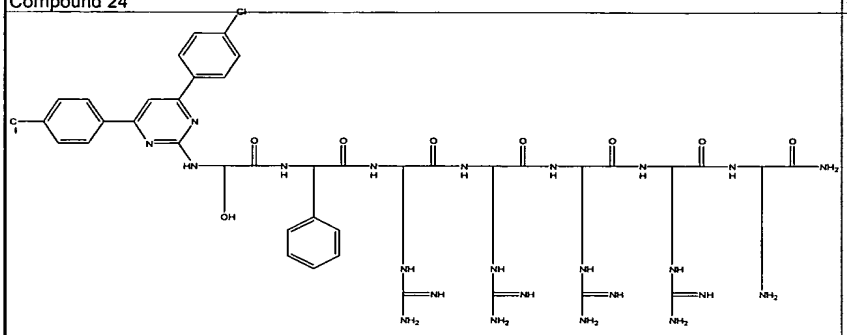
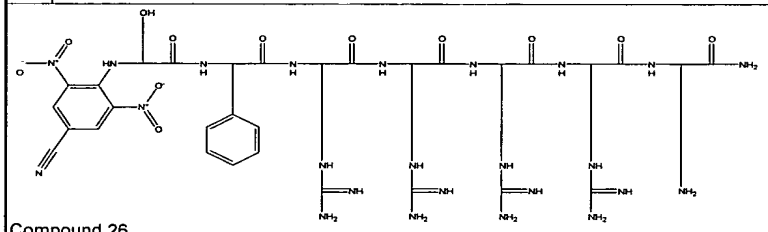
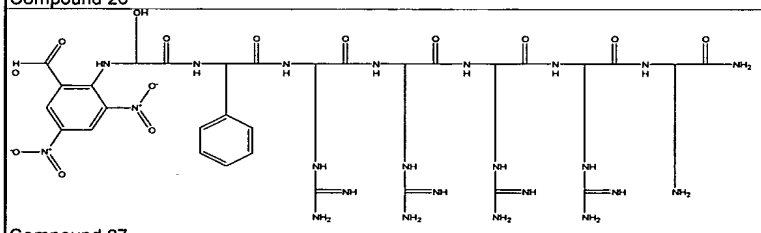
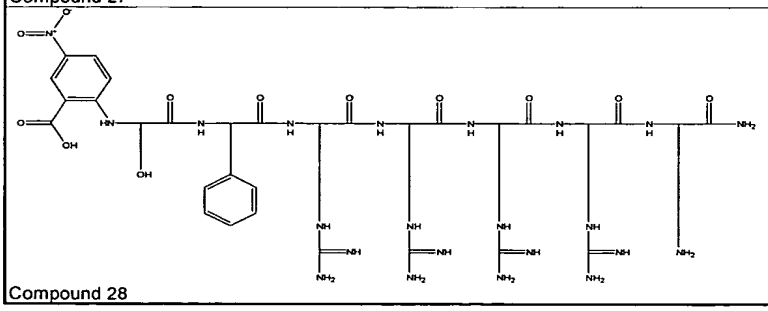
Table 3. Structures of 415 compounds tested. The % change observed in fluorescence intensity upon phosphorylation of the compound by protein kinase C is shown in the right column. Numbering of compounds for the purpose of this Table is arbitrary and does not correspond to compound numbers elsewhere in the specification. The compound labeled as compound 21 in Table 3 is the same as compound 2 in Example I, which is also shown in Scheme 2. Note that Table 3 does not include peptide 1 shown in Scheme 1 and compound 3 shown in Scheme 2.

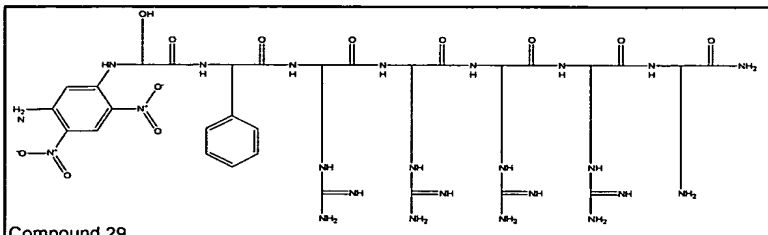
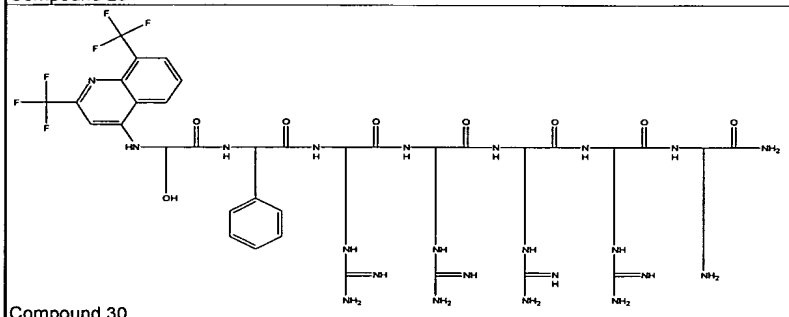
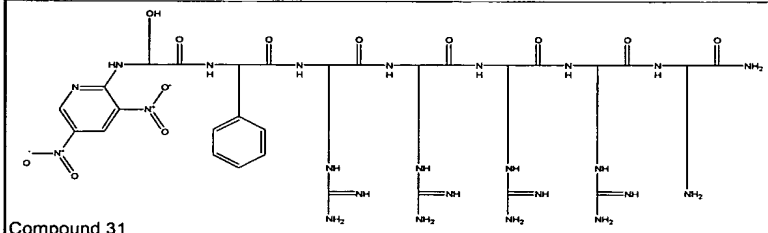
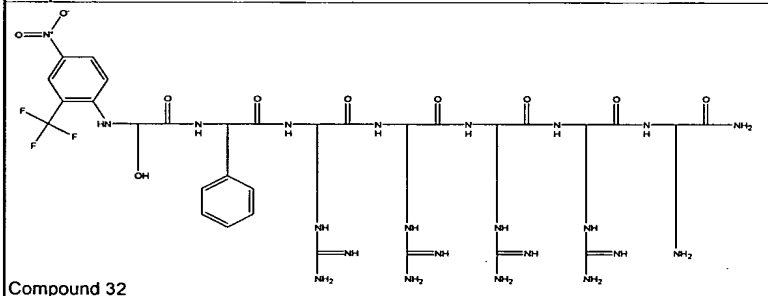
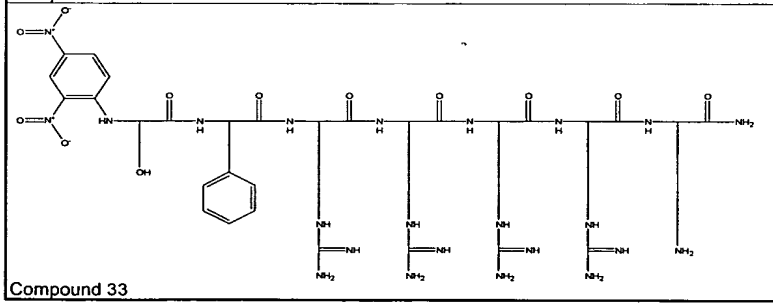
STRUCTURE	formula	molweight	%
Aldehydes to Amines			
 Compound 1	C49H83N21O9	1110.33	<10%
 Compound 2	C50H85N21O9	1124.3568	<10%
 Compound 3	C50H85N21O10	1140.3562	<10%
 Compound 4	C51H87N21O10	1154.383	<10%
 Compound 5	C51H88N22O8	1137.3988	<10%
 Compound 6	C51H88N22O9	1153.3982	<10%

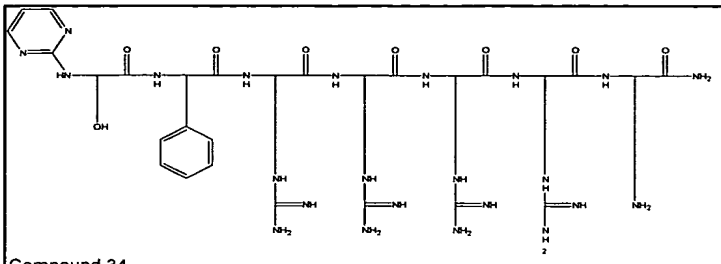
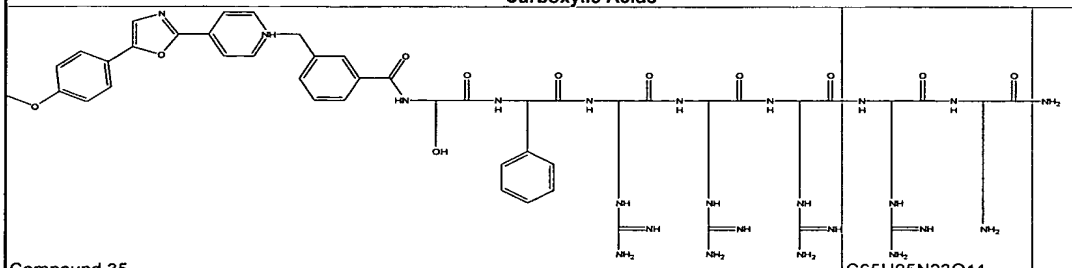
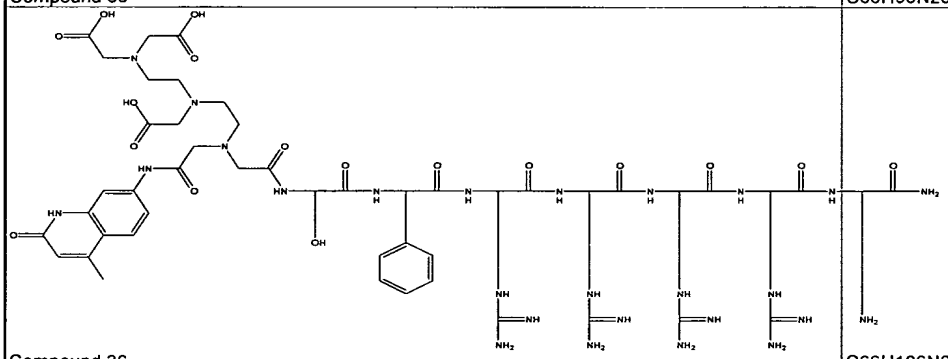
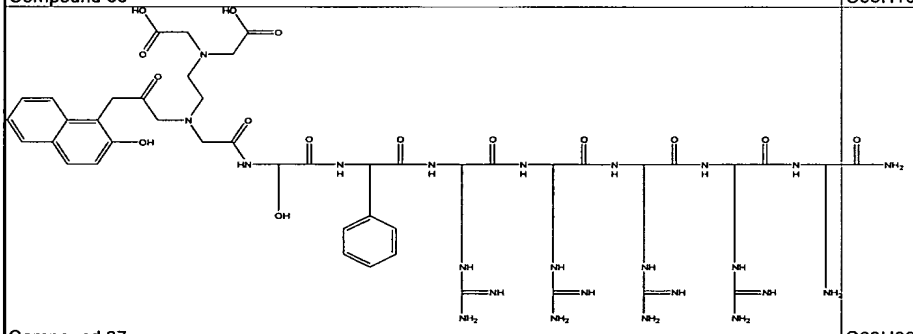
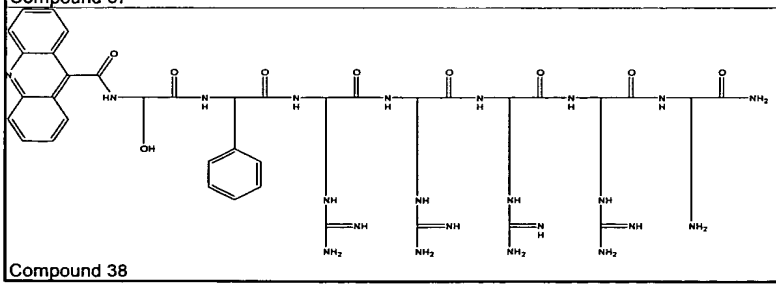
 Compound 7	C49H82N22O10	1139.3282	<10%
 Compound 8	C49H82N22O10	1139.3282	<10%
 Compound 9	C49H81N23O12	1184.3258	<10%
 Compound 10	C49H82N22O11	1155.3276	<10%
 Compound 11	C49H81N23O13	1200.3252	<10%
 Compound 12	C49H81Cl2N21O8	1163.2208	<10%

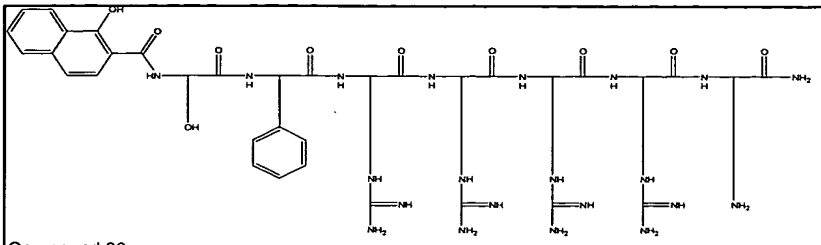
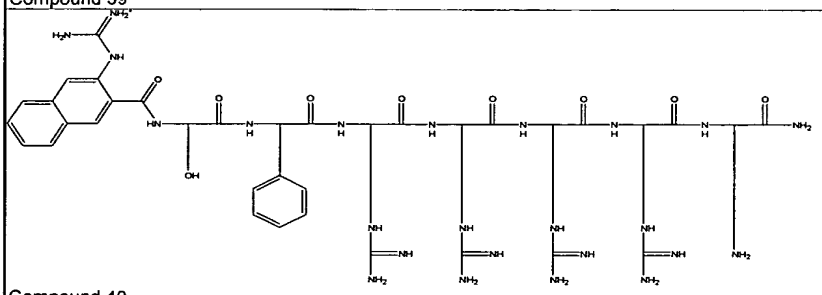
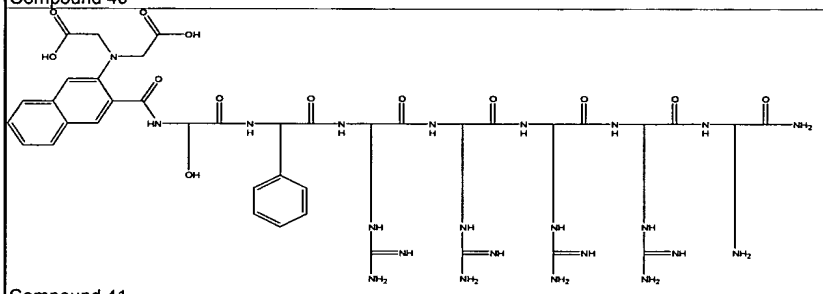
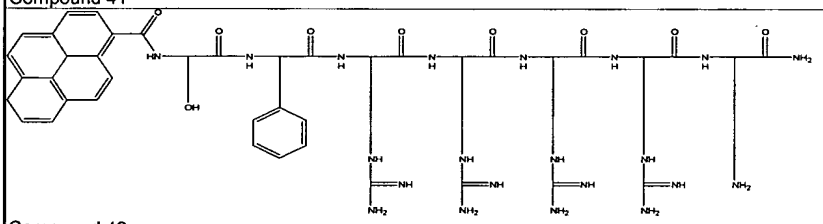
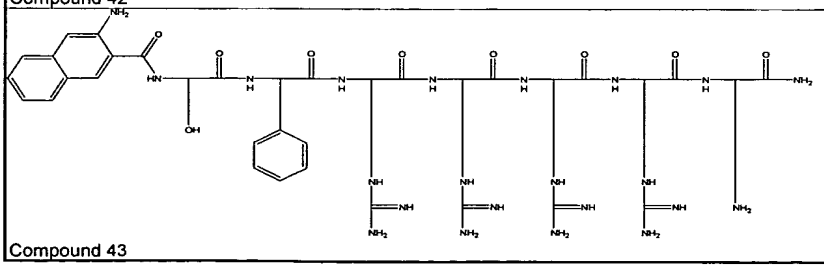
 Compound 13	C51H85N21O8	1120.3684	<10%
 Compound 14	C53H90N22O8	1163.4366	<10%
 Compound 15	C51H84N22O10	1165.366	<10%
 Compound 16	C57H89N21O8	1196.466	<10%
 Compound 17	C53H85N21O9	1160.3898	<10%
 Compound 18	C57H87N21O8	1194.4502	<10%

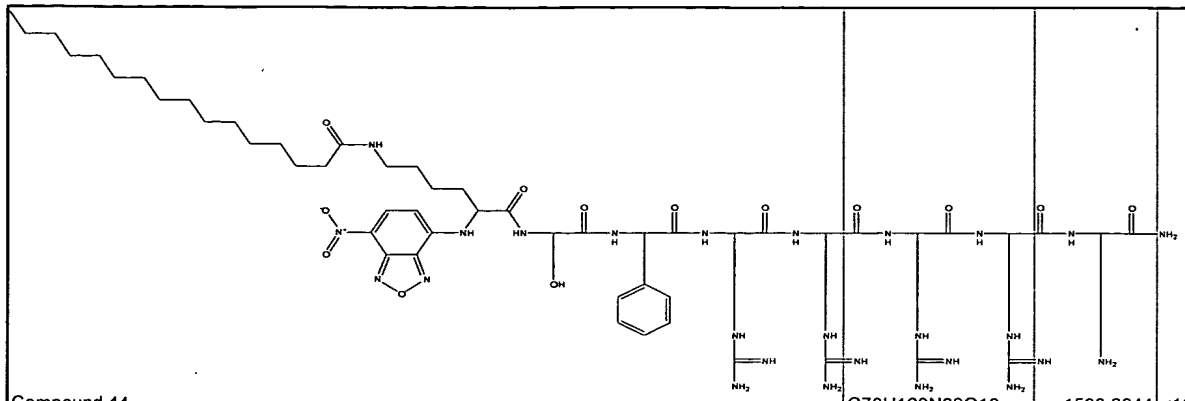
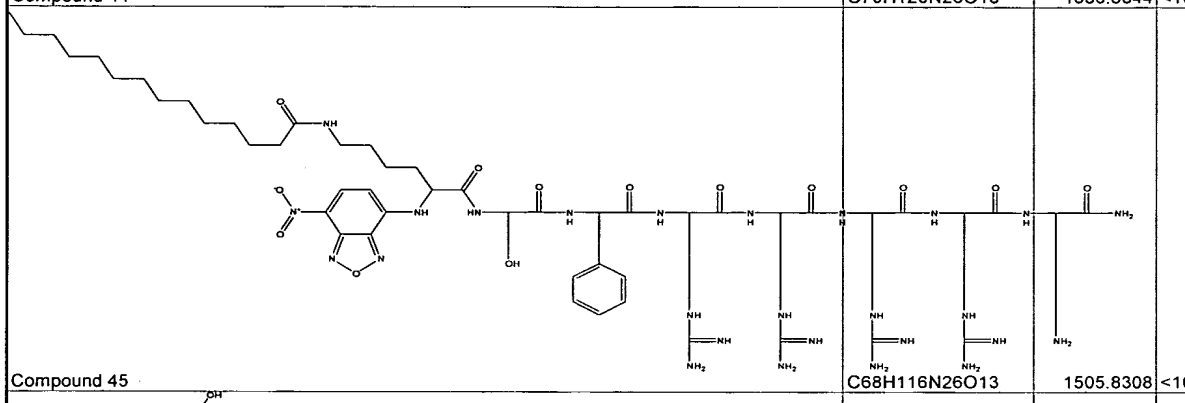
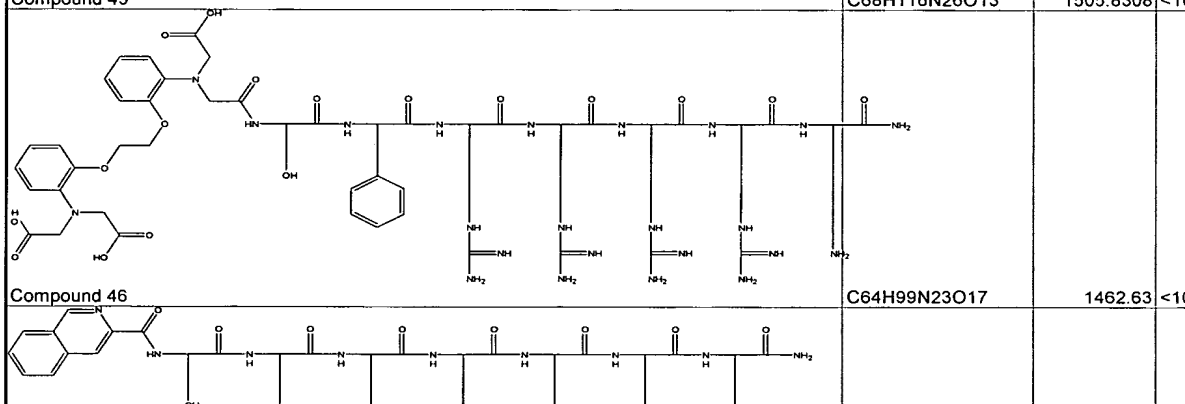
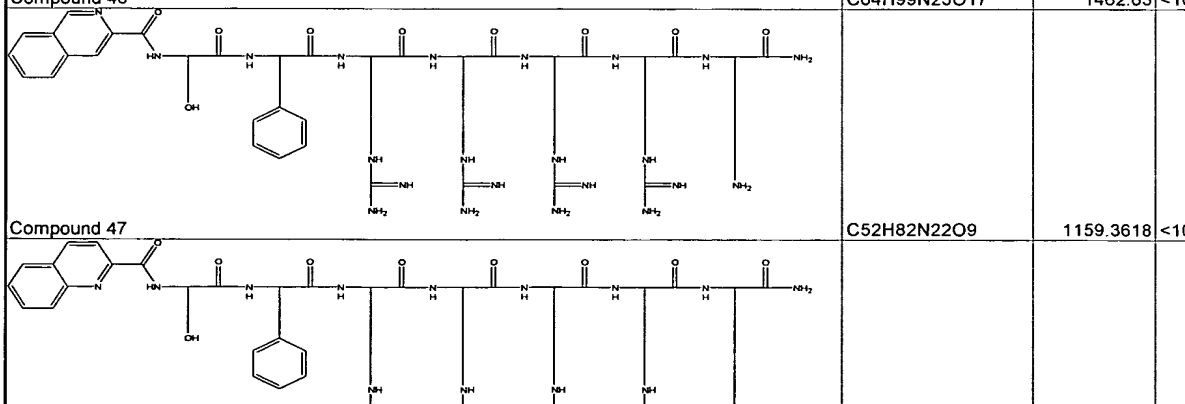
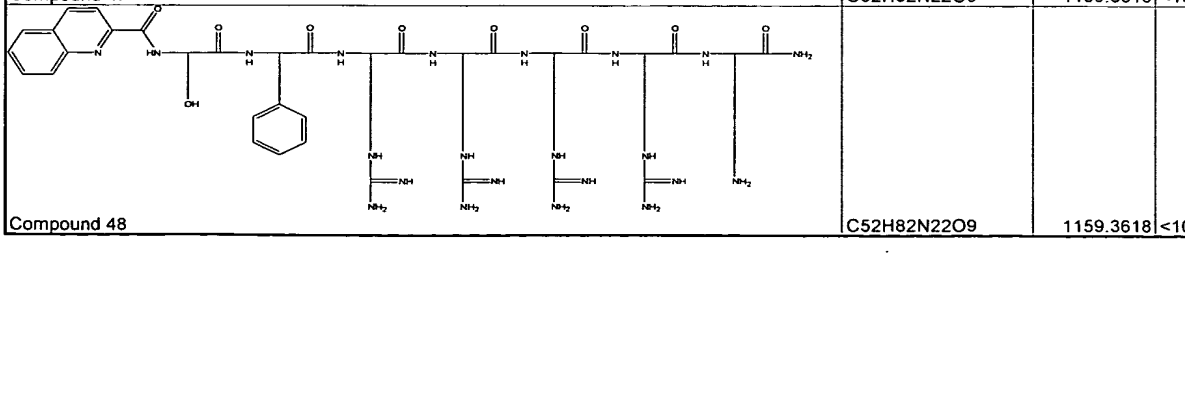
 Compound 19	C57H87N21O8	1194.4502	<10%
 Compound 20	C59H89N21O8	1220.488	<10%
Aryl halides			
 Compound 21	C48H78N24O11	1167.2984	150%
 Compound 22	C56H85N23O8	1208.4368	<10%
 Compound 23	C51H83N23O8	1146.366	<10%

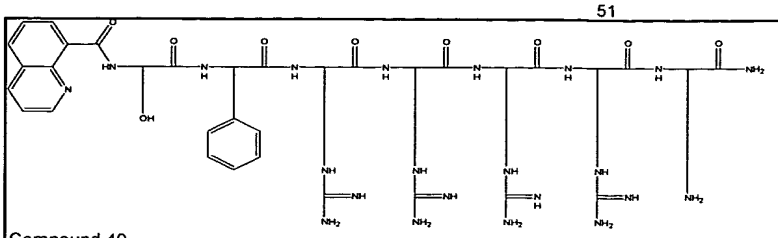
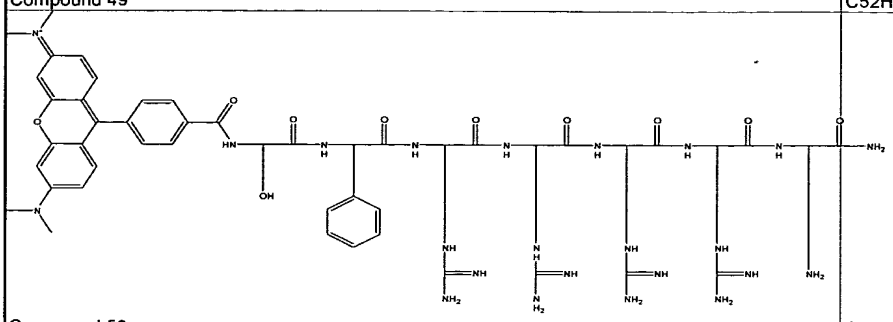
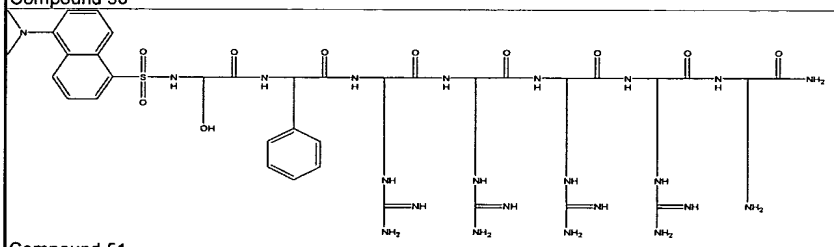
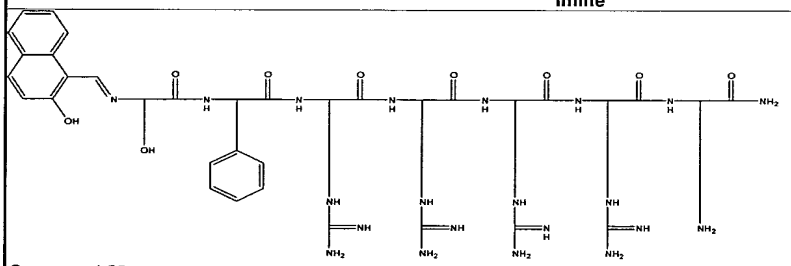
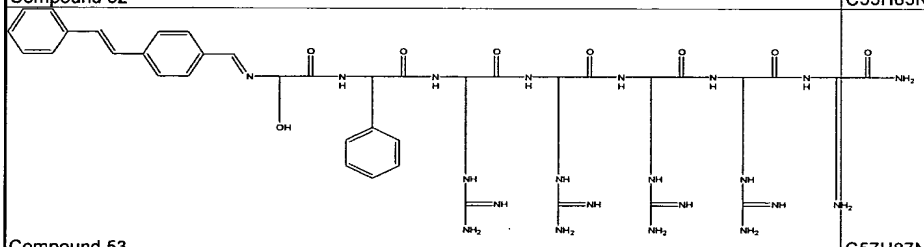
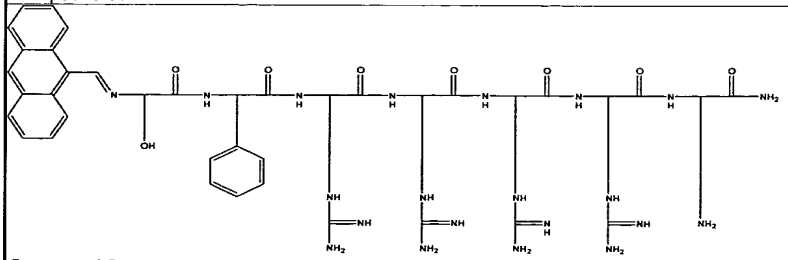
 Compound 24	C49H78Cl3N23O8	1223.6635	<10%
 Compound 25	C58H85Cl2N23O8	1303.3648	<10%
 Compound 26	C49H78N24O12	1195.3088	<10%
 Compound 27	C49H79N23O14	1214.3088	<10%
 Compound 28	C49H80N22O12	1169.3112	<10%

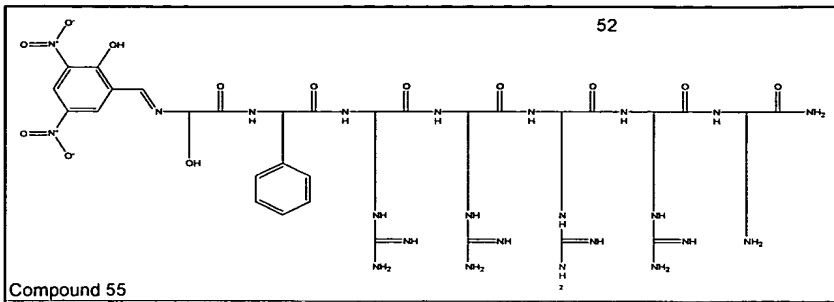
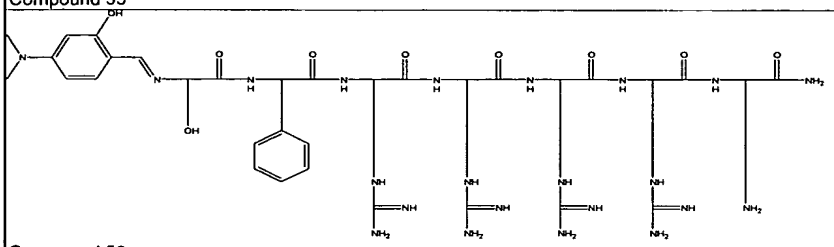
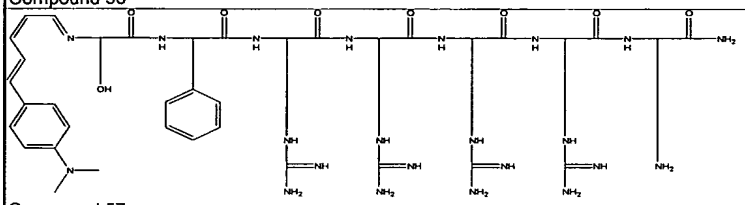
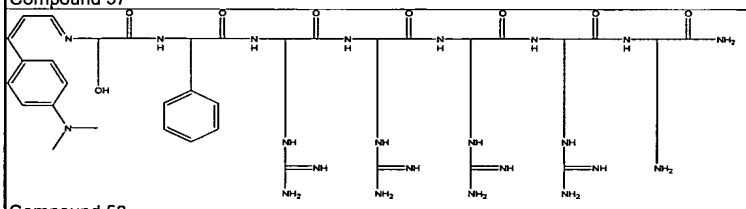
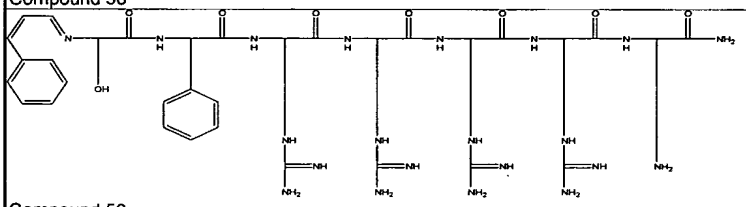
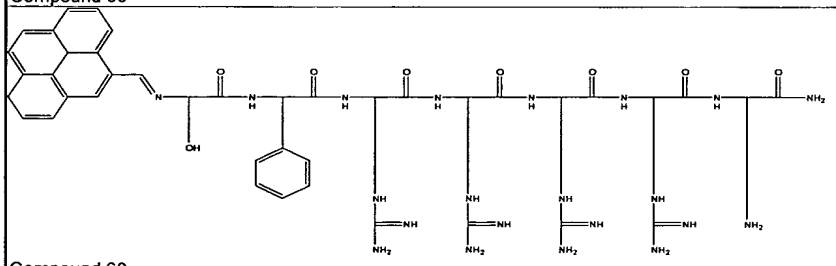
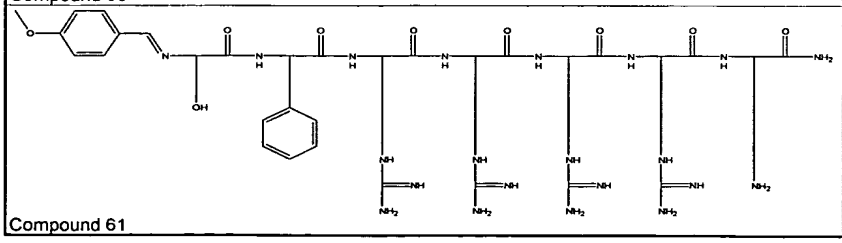
 Compound 29	C48H80N24O12	1185.3136	<10%
 Compound 30	C53H80F6N22O8	1267.348	<10%
 Compound 31	C47H78N24O12	1171.2868	<10%
 Compound 32	C49H79F3N22O10	1193.2997	<10%
 Compound 33	C48H79N23O12	1170.299	<10%

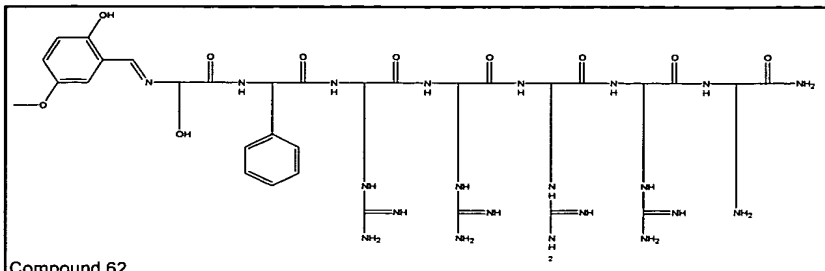
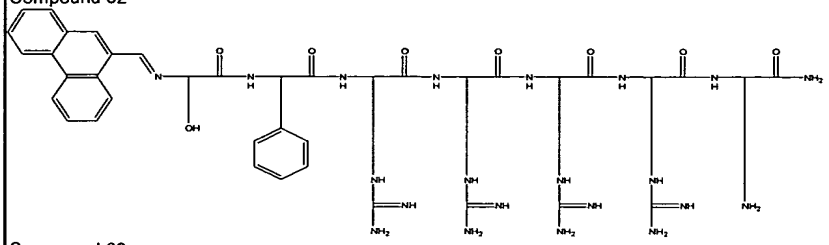
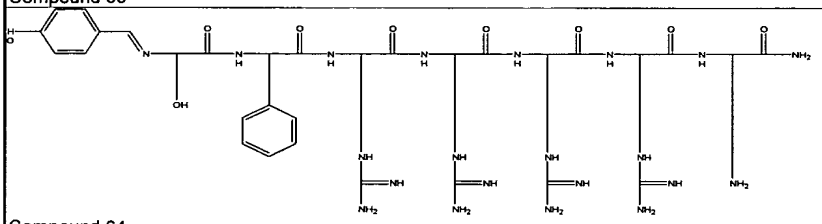
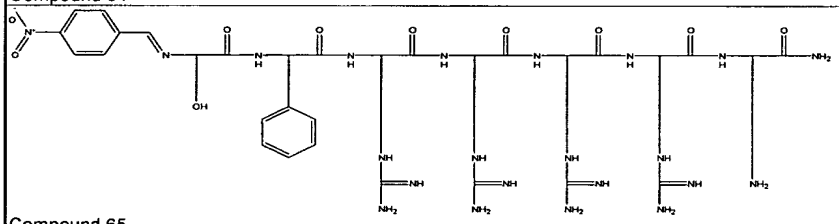
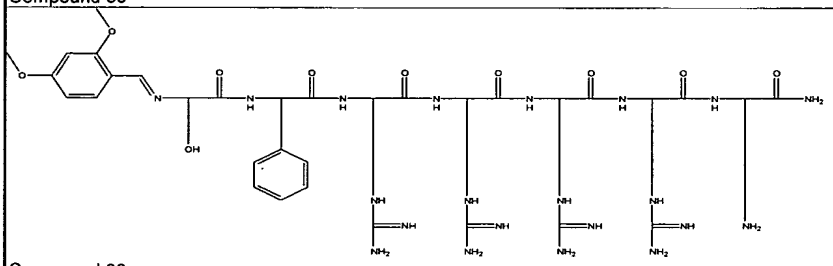
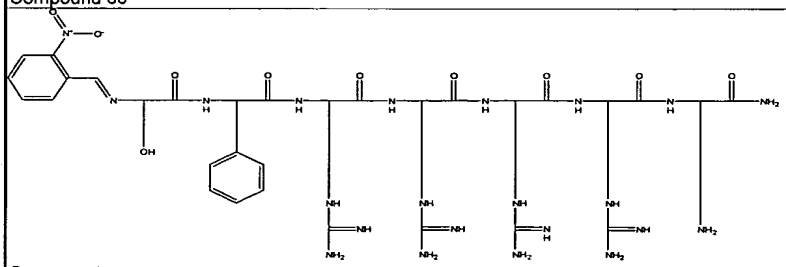
 Compound 34	C46H79N23O8	1082.2794	<10%
Carboxylic Acids			
 Compound 35	C65H95N23O11	1374.613	<10%
 Compound 36	C66H106N26O17	1535.7274	<10%
 Compound 37	C63H99N23O15	1418.6202	<10%
 Compound 38	C56H84N22O9	1209.4216	20%

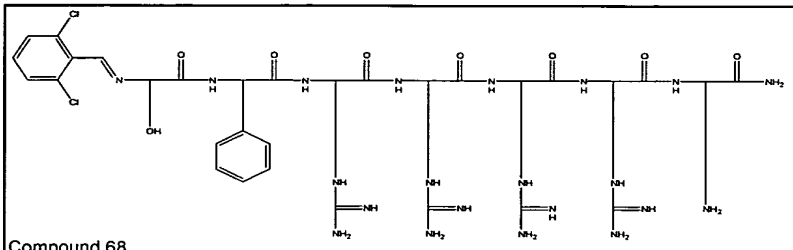
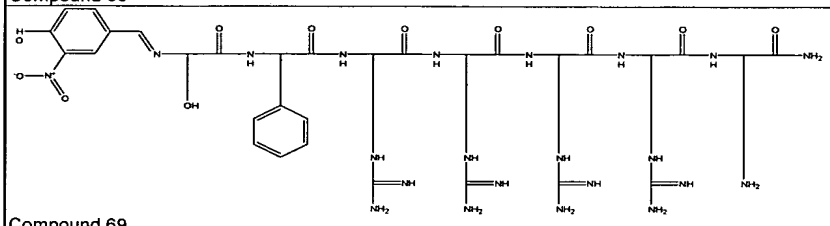
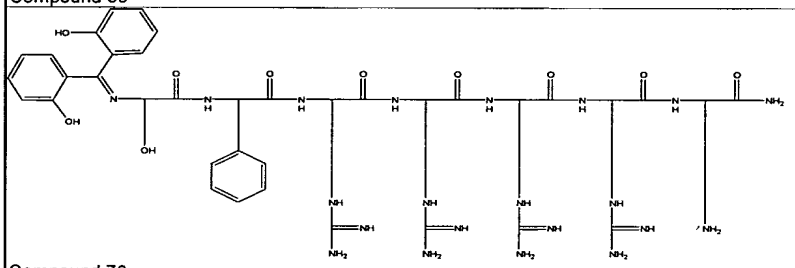
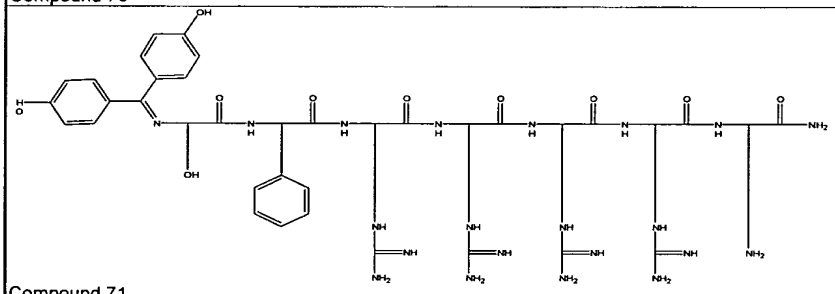
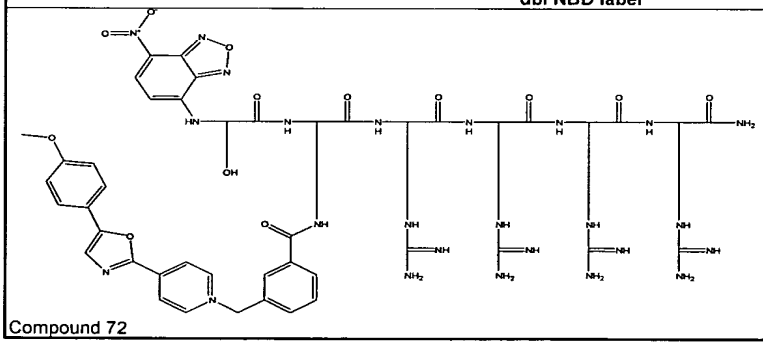
 Compound 39	C53H83N21O10	1174.3734	<10%
 Compound 40	C54H87N24O9+	1216.4367	<10%
 Compound 41	C57H88N22O13	1289.4618	<10%
 Compound 42	C59H87N21O9	1234.4716	<10%
 Compound 43	C53H84N22O9	1173.3886	<10%

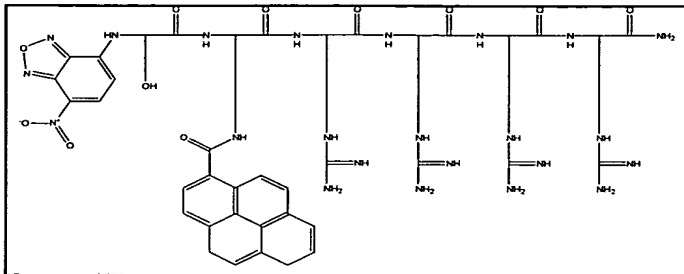
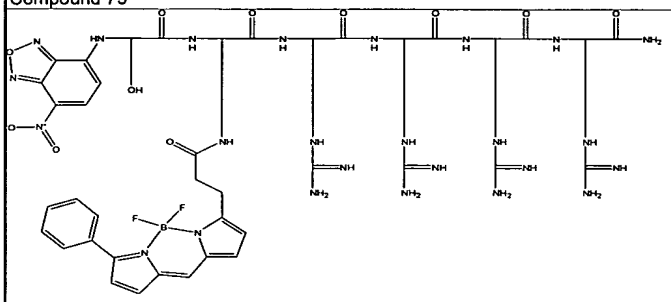
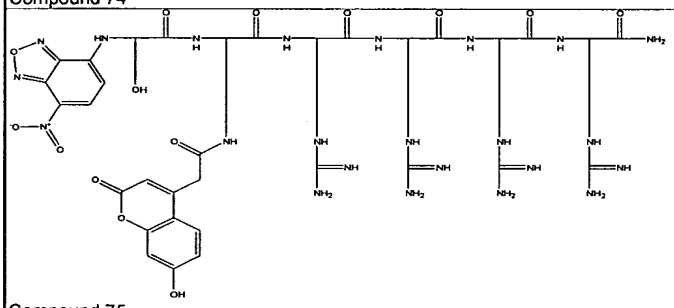
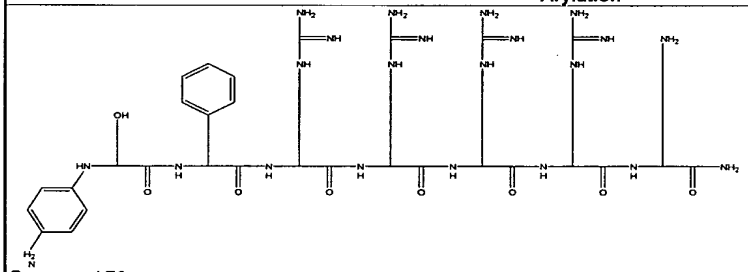
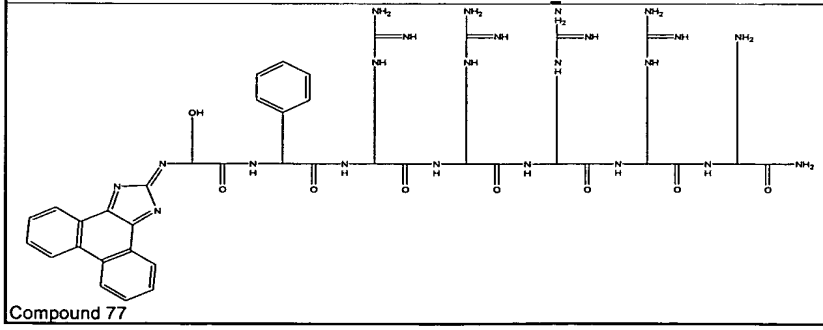
 Compound 44	C70H120N26O13	1533.8844	<10%
 Compound 45	C68H116N26O13	1505.8308	<10%
 Compound 46	C64H99N23O17	1462.63	<10%
 Compound 47	C52H82N22O9	1159.3618	<10%
 Compound 48	C52H82N22O9	1159.3618	<10%

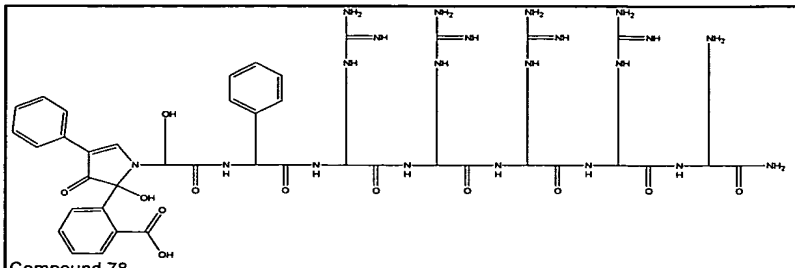
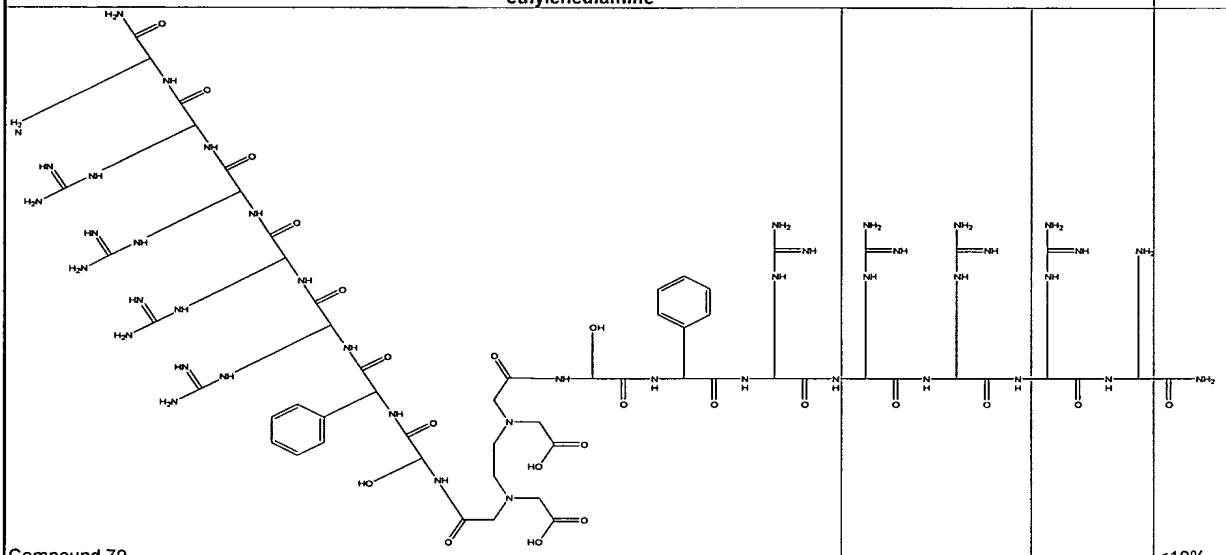
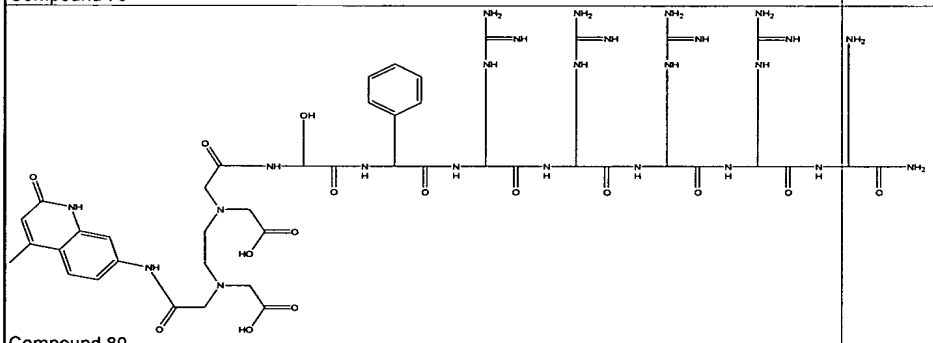
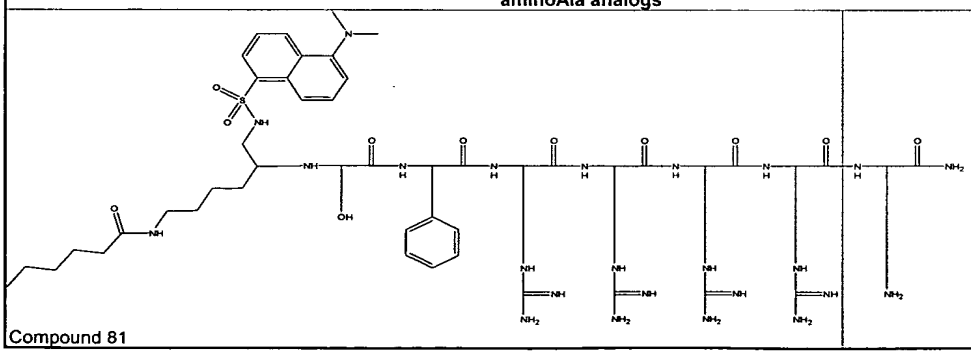
 Compound 49	C52H82N22O9	1159.3618	<10%
 Compound 50	C66H98N23O10+	1373.6483	<10%
 Compound 51	C54H88N22O10S	1237.4906	20%
Imine			
 Compound 52	C53H83N21O9	1158.374	<10%
 Compound 53	C57H87N21O8	1194.4502	<10%
 Compound 54	C57H85N21O8	1192.4344	<10%

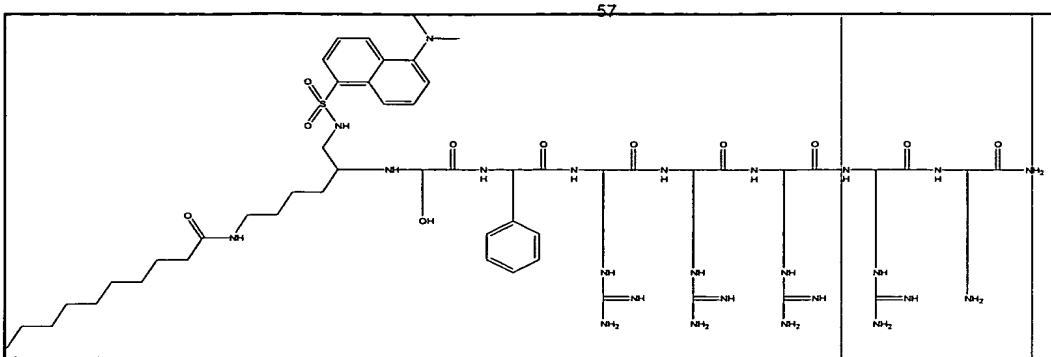
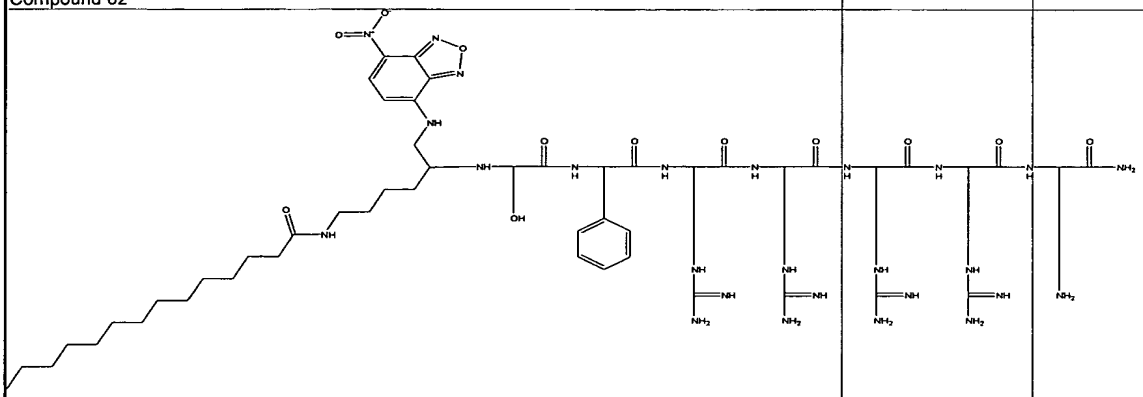
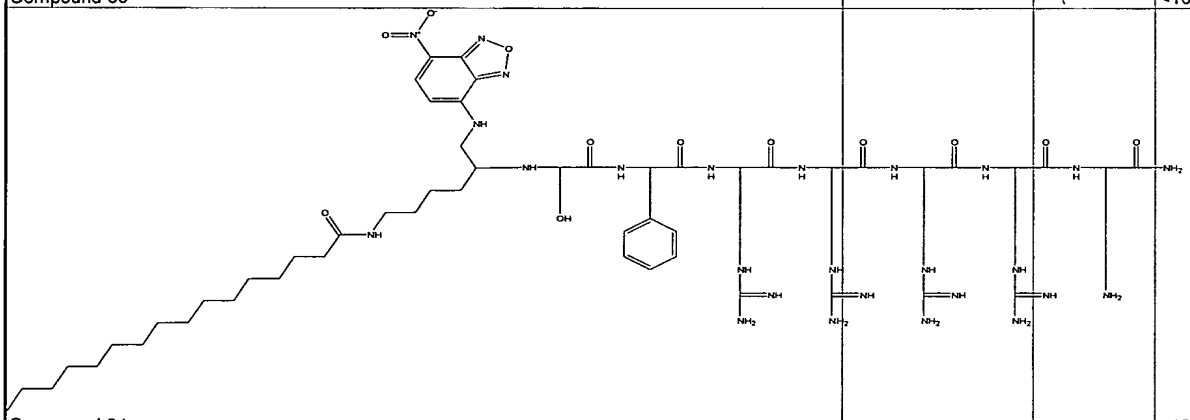
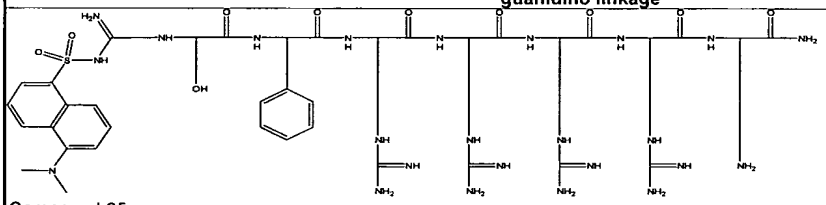
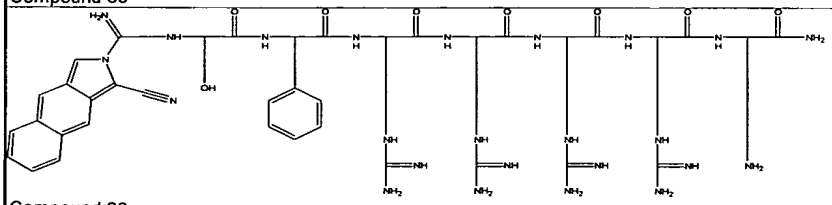
 Compound 55	C49H79N23O13	1198.3094	<10%
 Compound 56	C51H86N22O9	1151.3824	<10%
 Compound 57	C55H90N22O8	1187.4586	<10%
 Compound 58	C53H88N22O8	1161.4208	<10%
 Compound 59	C51H83N21O8	1118.3526	<10%
 Compound 60	C59H87N21O8	1218.4722	<10%
 Compound 61	C50H83N21O9	1122.341	<10%

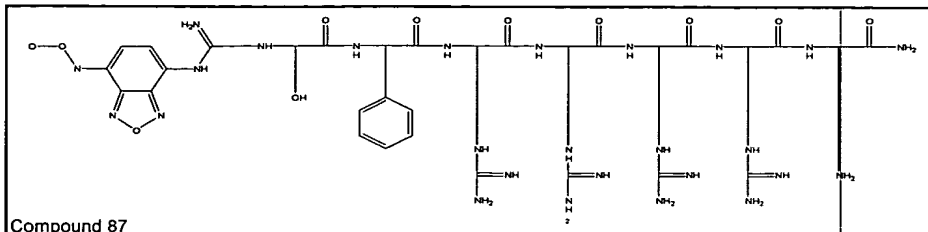
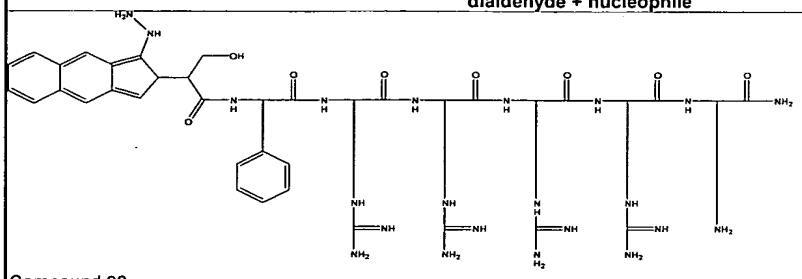
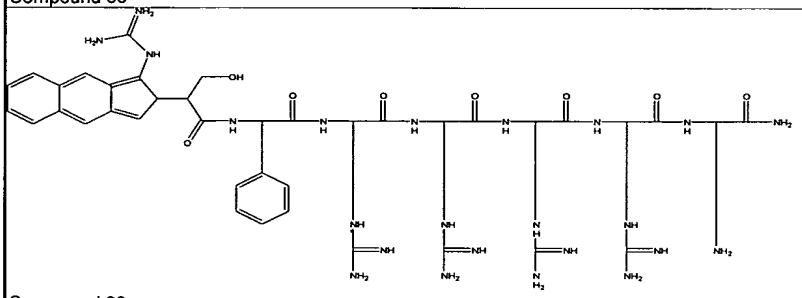
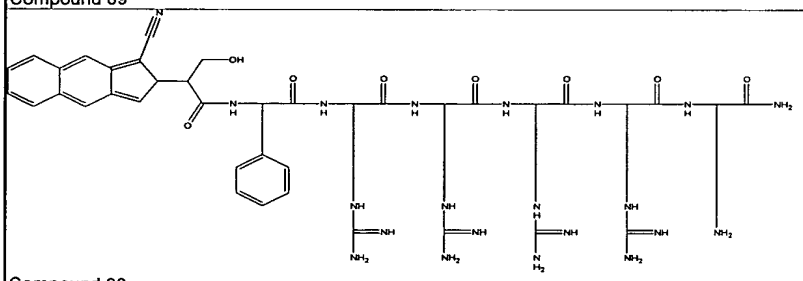
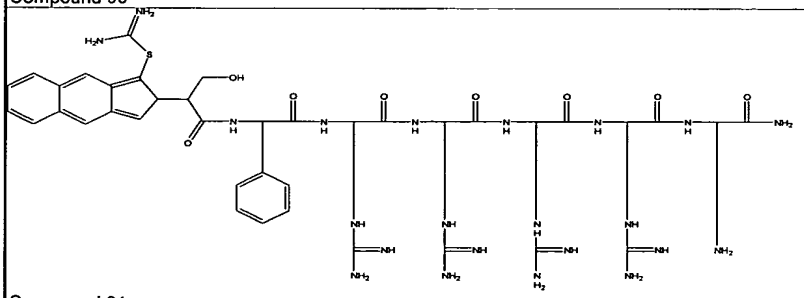
 Compound 62	C50H83N21O10	1138.3404	<10%
 Compound 63	C57H85N21O8	1192.4344	<10%
 Compound 64	C49H81N21O9	1108.3142	<10%
 Compound 65	C49H80N22O10	1137.3124	<10%
 Compound 66	C51H85N21O10	1152.3672	<10%
 Compound 67	C49H80N22O10	1137.3124	<10%

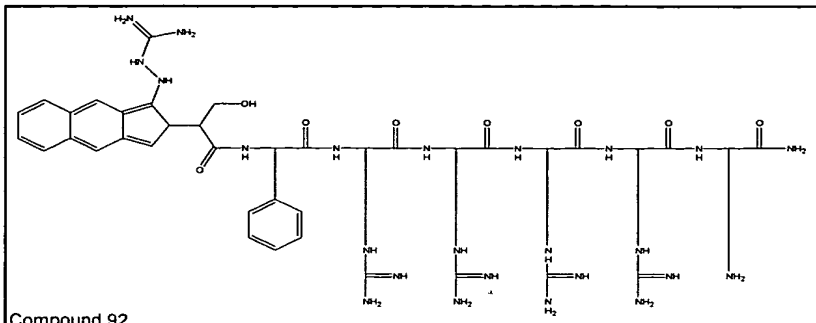
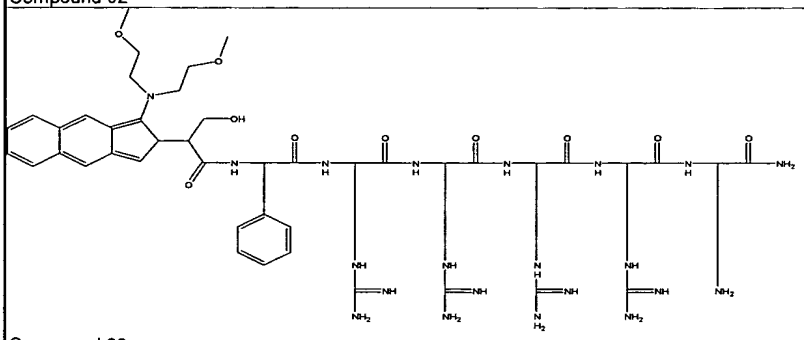
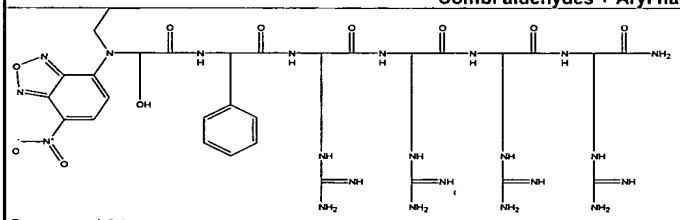
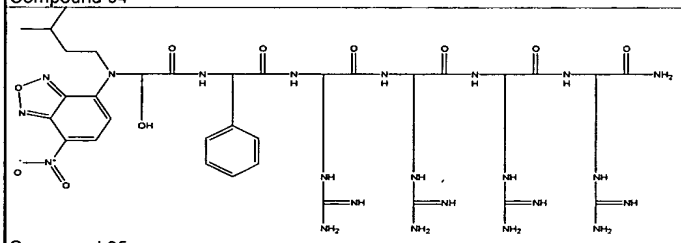
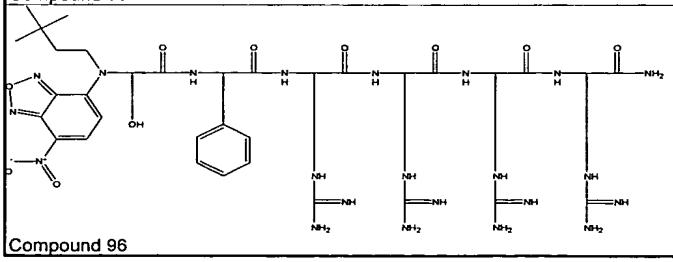
 Compound 68	C49H79Cl2N21O8	1161.205	<10%
 Compound 69	C49H80N22O11	1153.3118	<10%
 Compound 70	C55H85N21O10	1200.4112	<10%
 Compound 71	C55H85N21O10	1200.4112	<10%
dbl NBD label			
 Compound 72			<10%

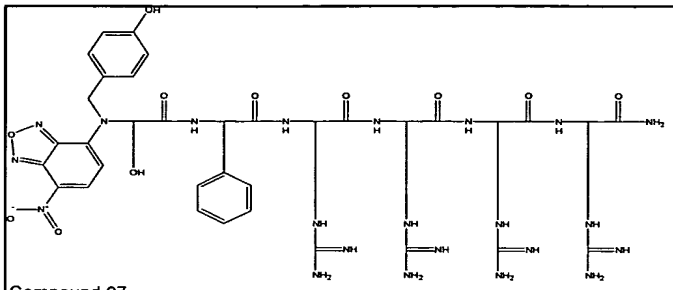
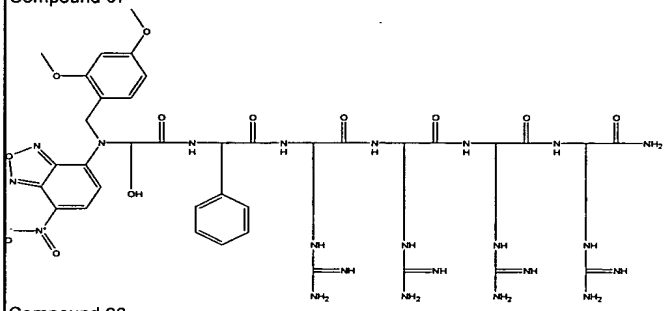
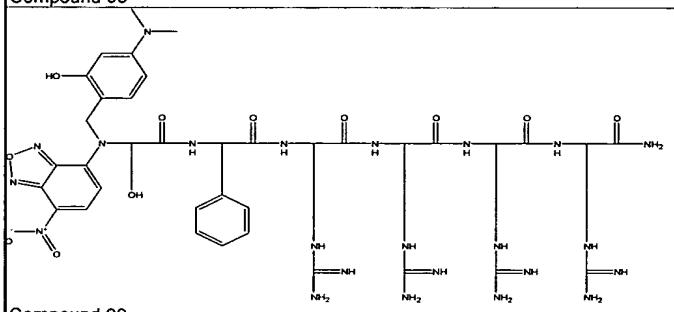
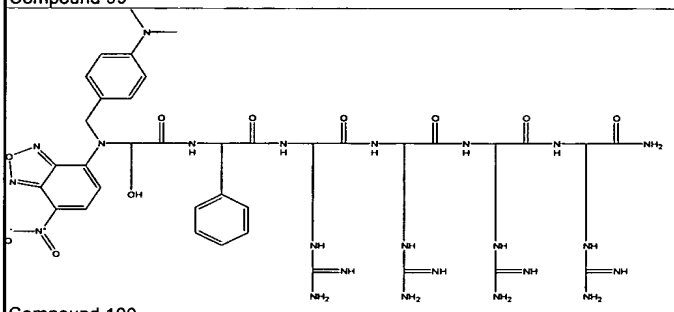
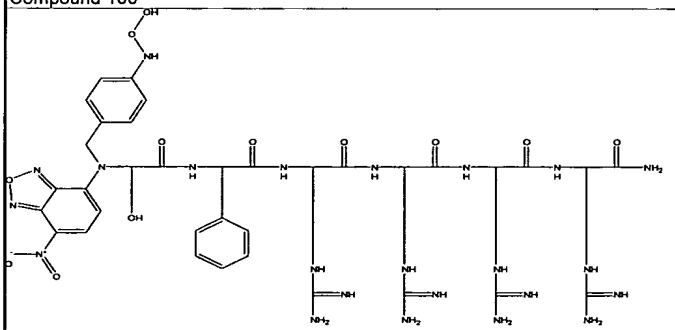
 Compound 73			<10%
 Compound 74			<10%
 Compound 75			<10%
Arylation			
 Compound 76			<10%
ketimine_fluorescamine			
 Compound 77			<10%

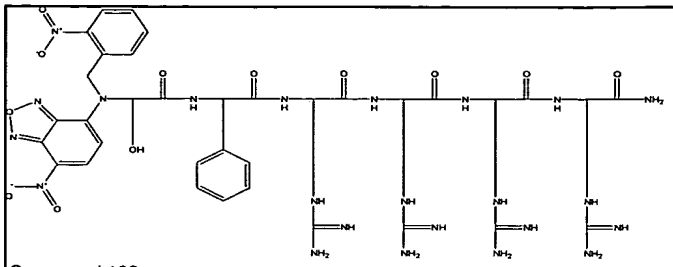
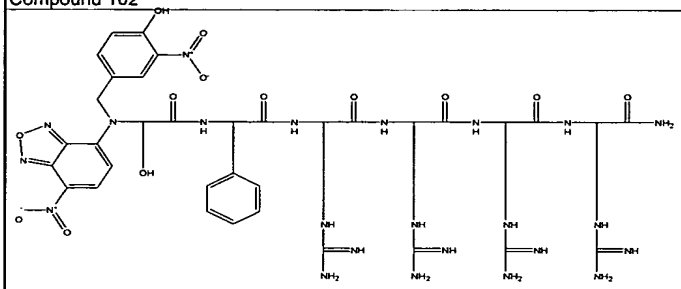
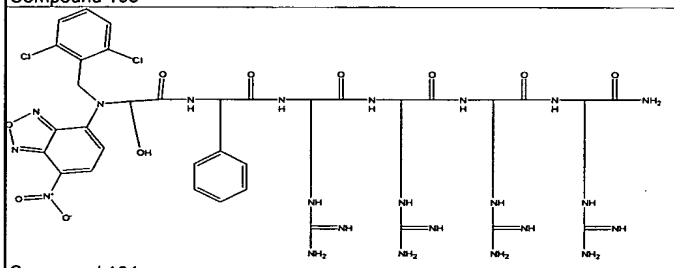
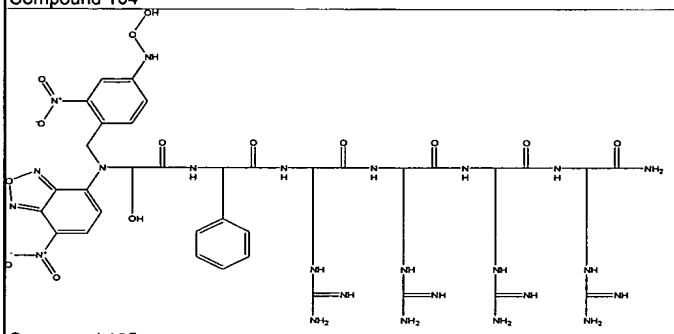
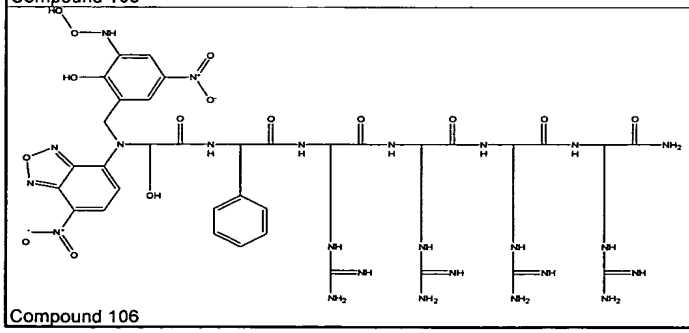
 Compound 78			<10%
ethylenediamine			
 Compound 79			<10%
 Compound 80			<10%
aminoAla analogs			
 Compound 81			<10%

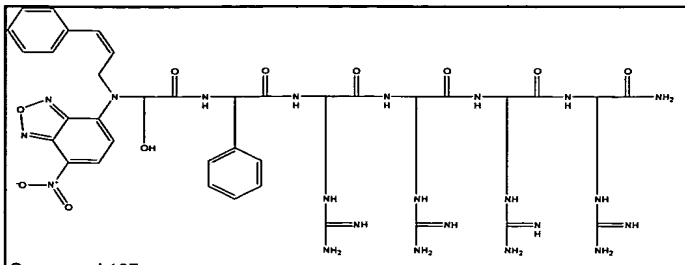
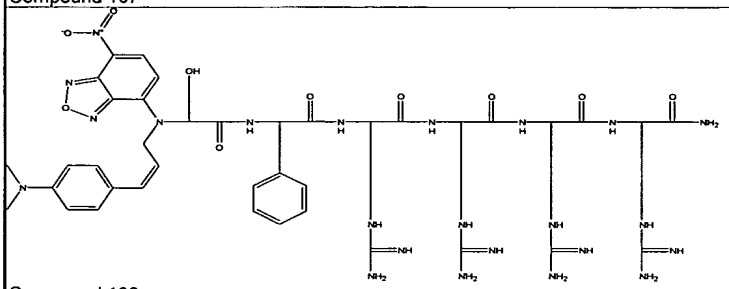
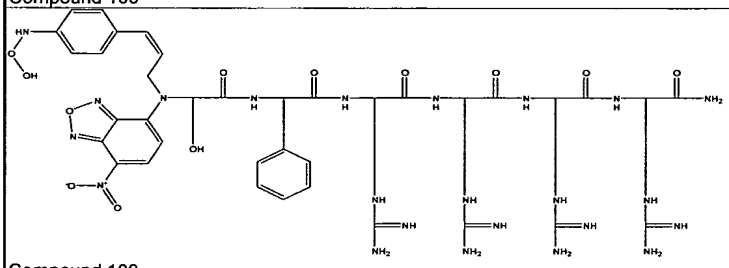
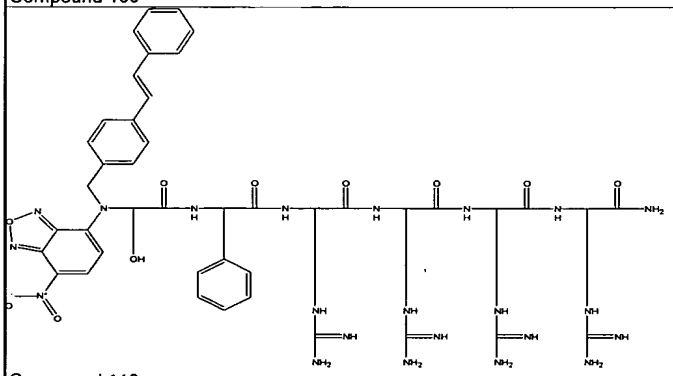
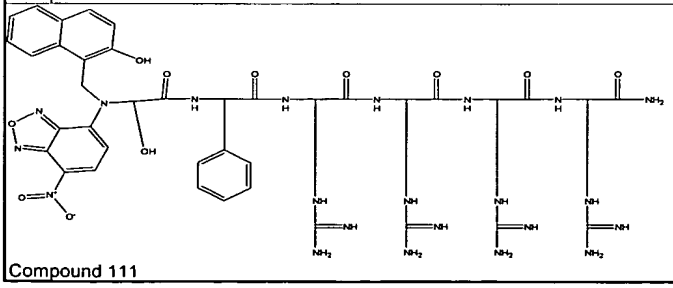
 Compound 82		<10%
 Compound 83		<10%
 Compound 84		<10%
guanidino linkage		
 Compound 85		<10%
 Compound 86		<10%

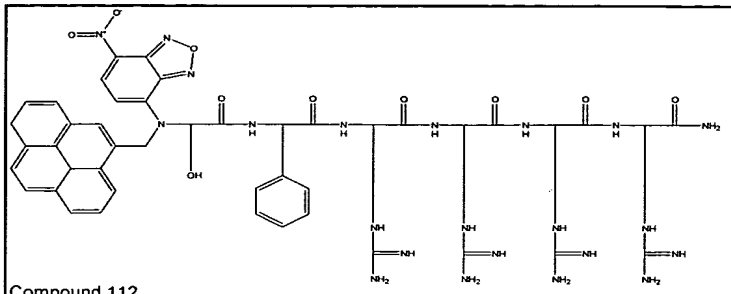
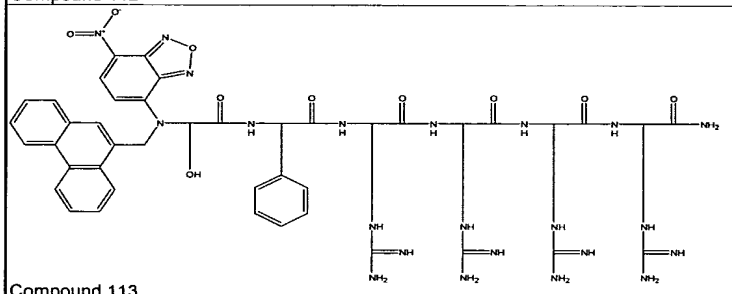
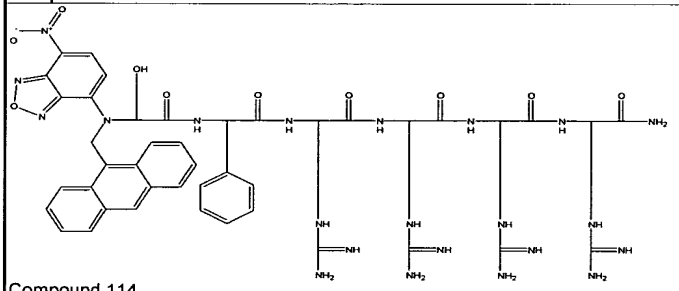
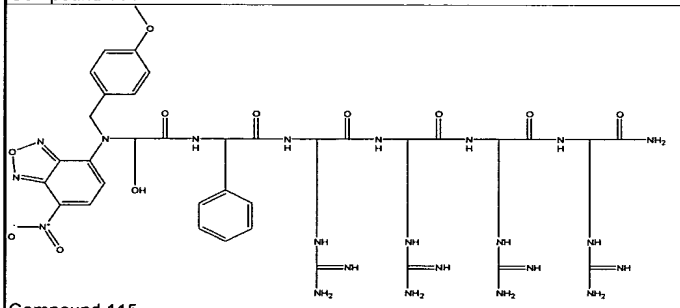
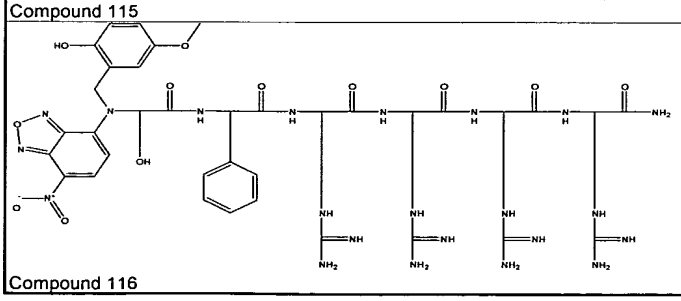
 Compound 87			<10%
dialdehyde + nucleophile			
 Compound 88			<10%
 Compound 89			<10%
 Compound 90			<10%
 Compound 91			<10%

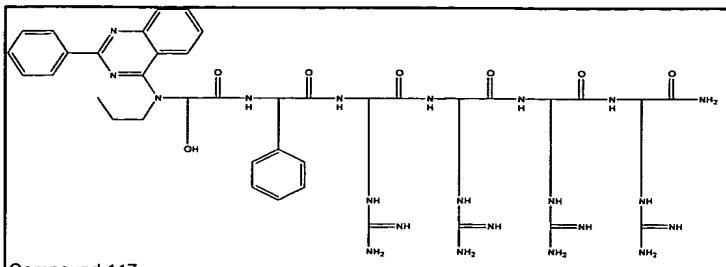
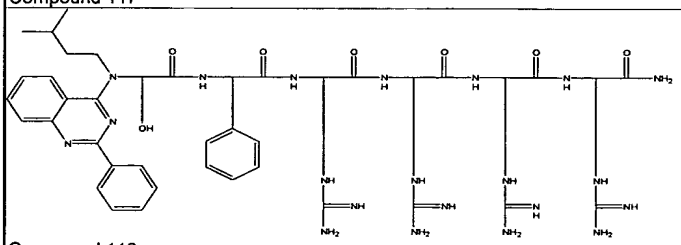
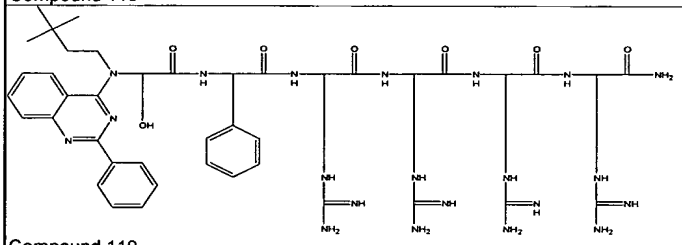
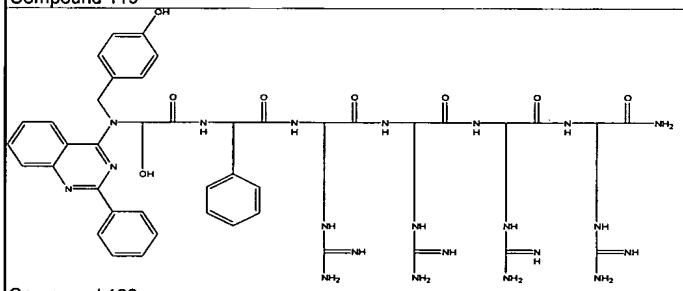
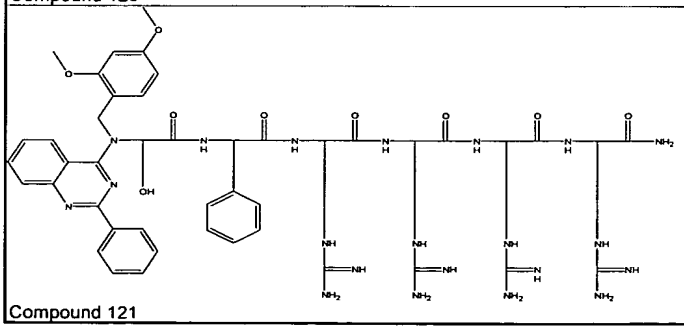
 Compound 92			<10%
 Compound 93			<10%
Combi aldehydes + Aryl halides			
 Compound 94	C45H72N22O10	1081.2052	<10%
 Compound 95	C47H76N22O10	1109.2588	<10%
 Compound 96	C48H78N22O10	1123.2856	<10%

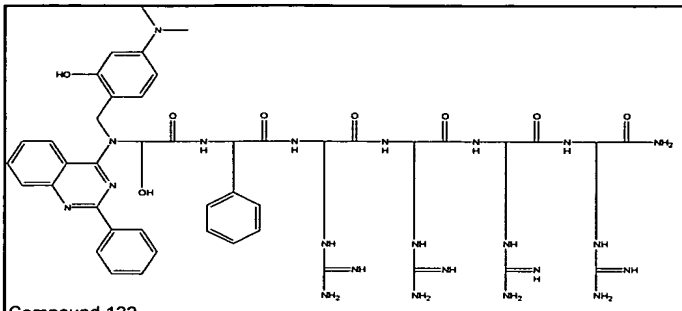
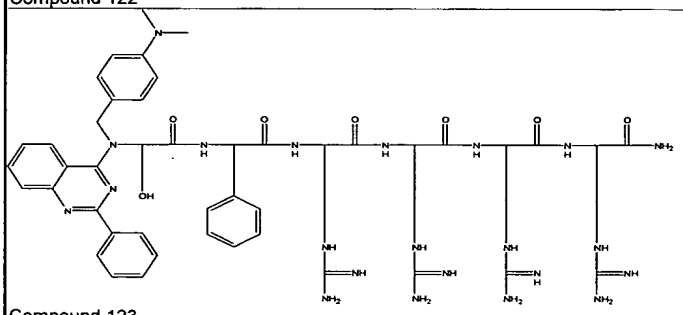
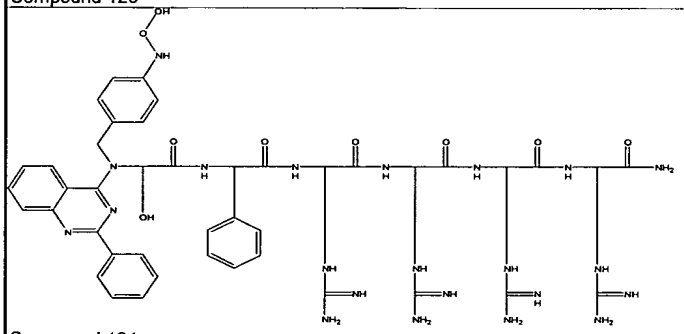
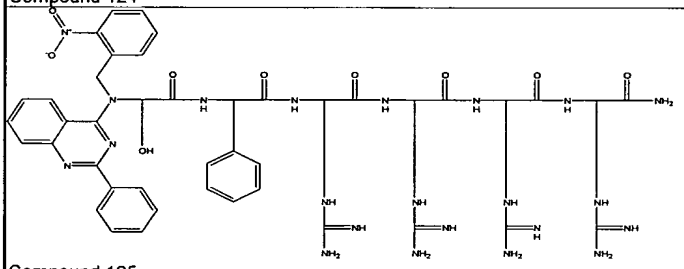
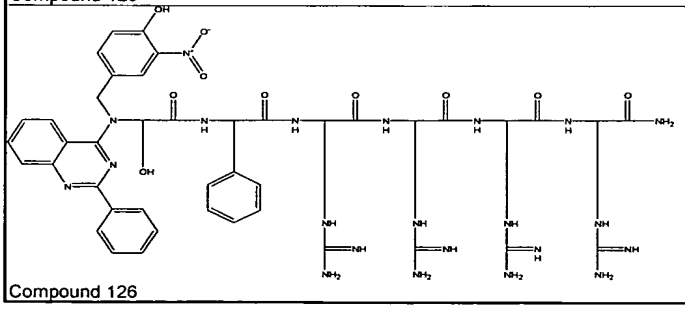
 Compound 97	C49H72N22O11	1145.2486	<10%
 Compound 98	C51H76N22O12	1189.3016	<10%
 Compound 99	C51H77N23O11	1188.3168	<10%
 Compound 100	C51H77N23O10	1172.3174	<10%
 Compound 101	C49H73N23O12	1176.2626	<10%

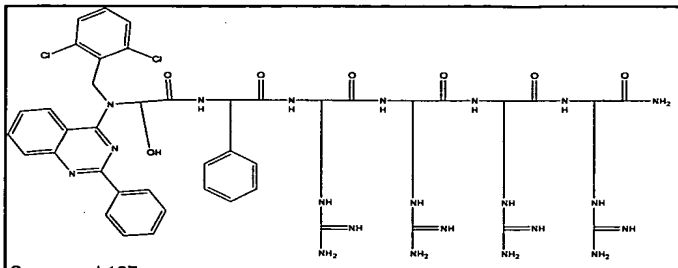
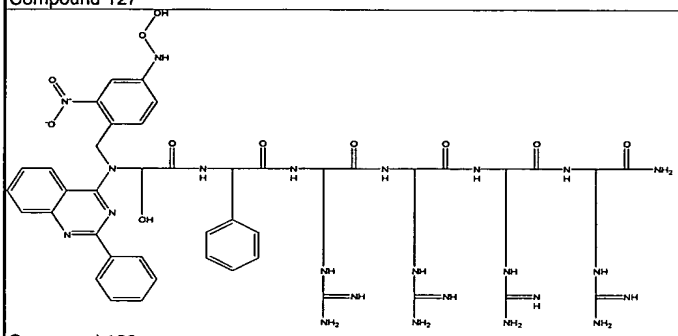
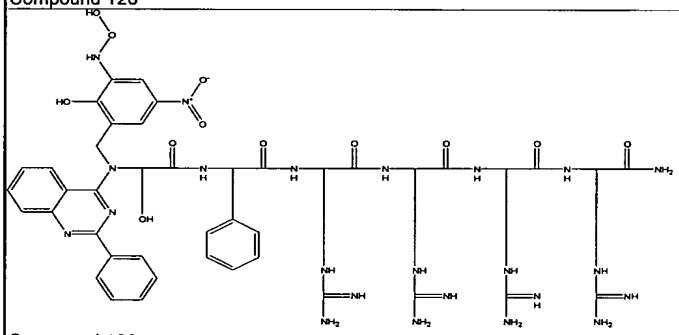
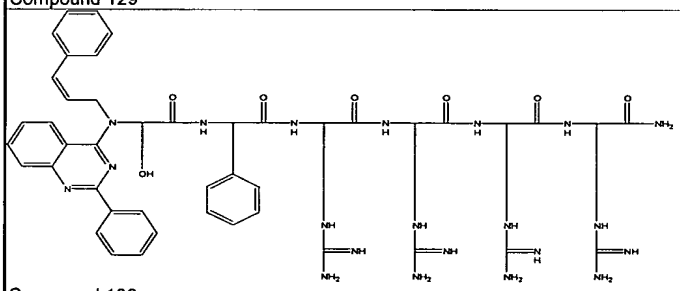
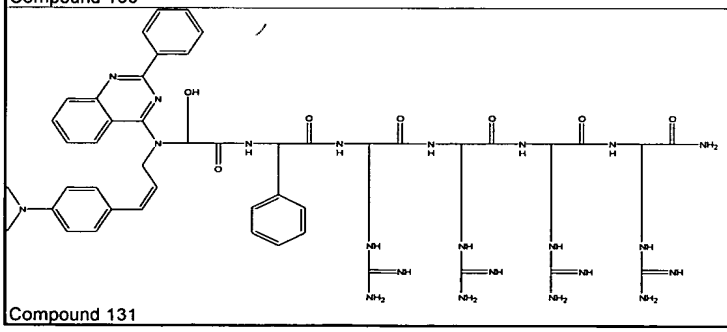
 Compound 102	C49H71N23O12	1174.2468	<10%
 Compound 103	C49H71N23O13	1190.2462	<10%
 Compound 104	C49H70Cl2N22O10	1198.1394	<10%
 Compound 105	C49H72N24O14	1221.2602	<10%
 Compound 106	C49H72N24O15	1237.2596	<10%

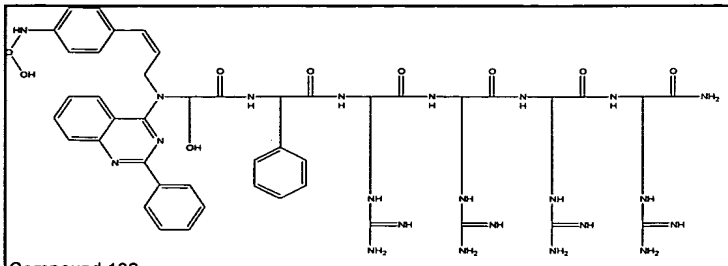
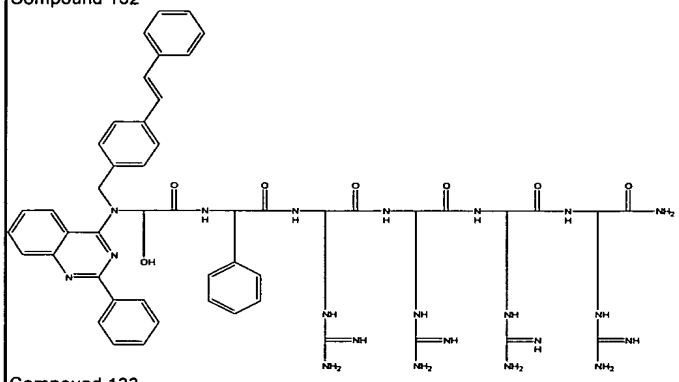
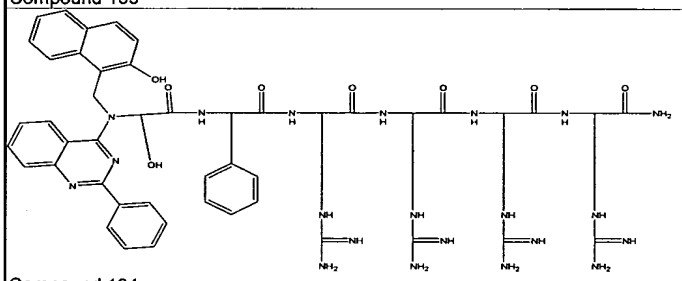
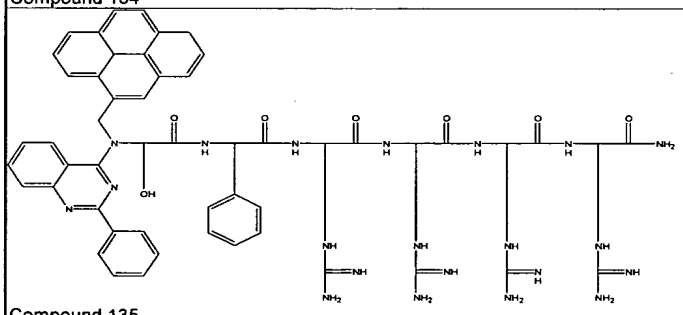
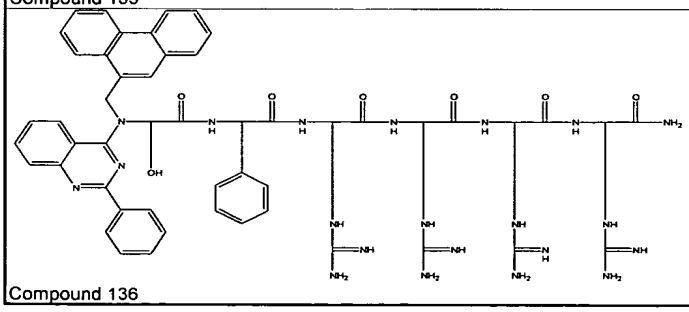
 Compound 107	C51H74N22O10	1155.287	<10%
 Compound 108	C53H79N23O10	1198.3552	<10%
 Compound 109	C51H75N23O12	1202.3004	<10%
 Compound 110	C57H78N22O10	1231.3846	<10%
 Compound 111	C53H74N22O11	1195.3084	<10%

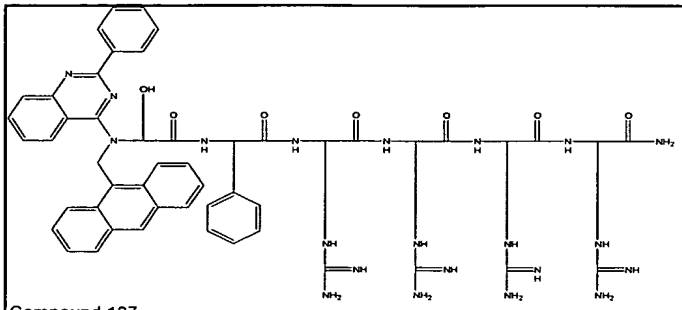
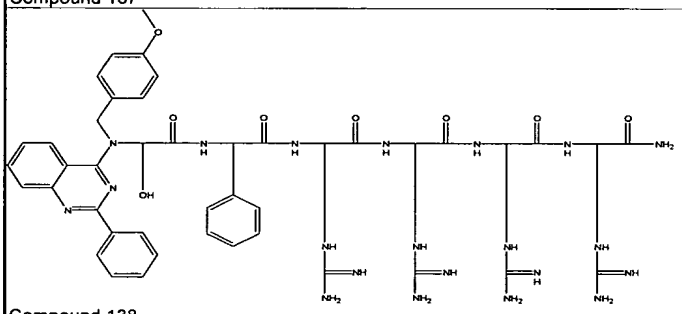
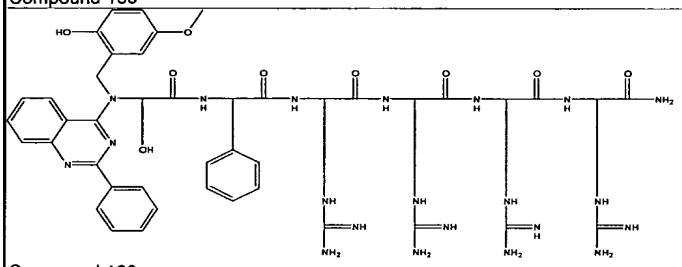
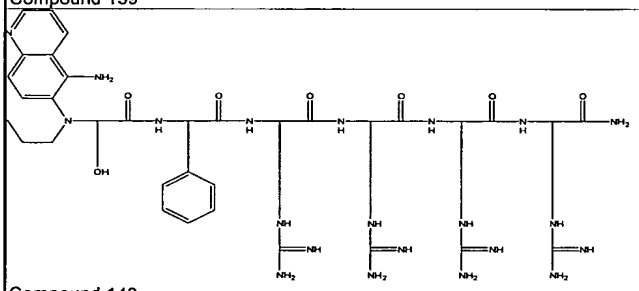
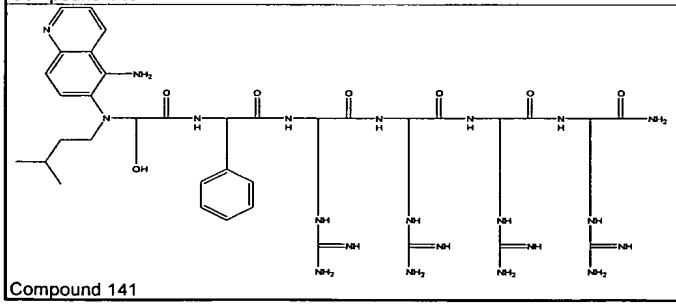
 Compound 112	C59H78N22O10	1255.4066	<10%
 Compound 113	C57H76N22O10	1229.3688	<10%
 Compound 114	C57H76N22O10	1229.3688	<10%
 Compound 115	C50H74N22O11	1159.2754	<10%
 Compound 116	C50H74N22O12	1175.2748	<10%

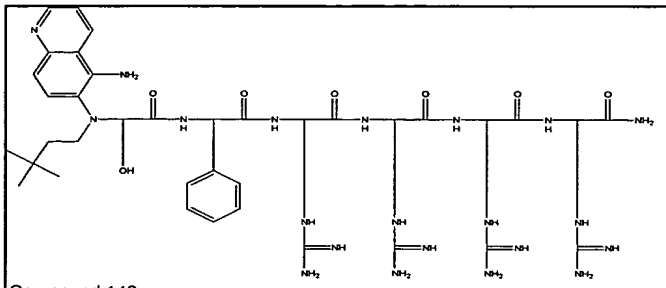
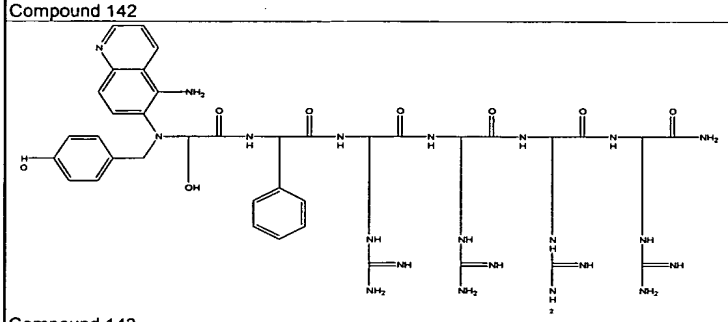
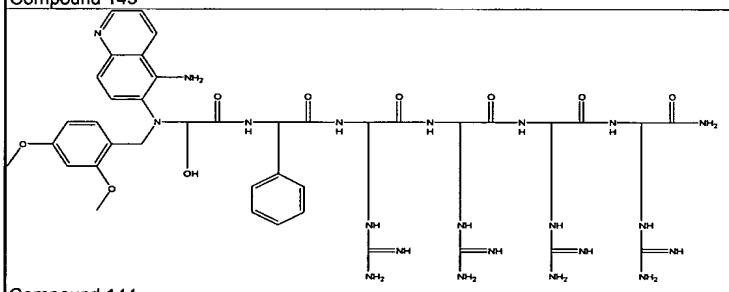
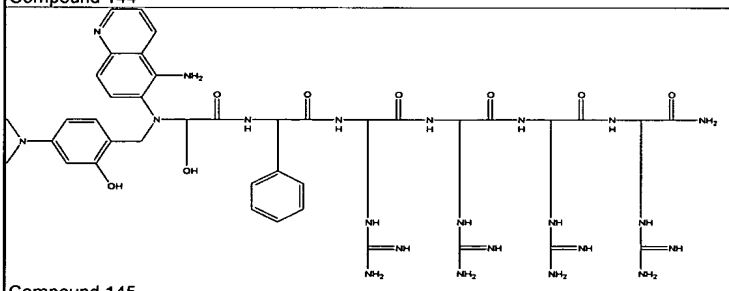
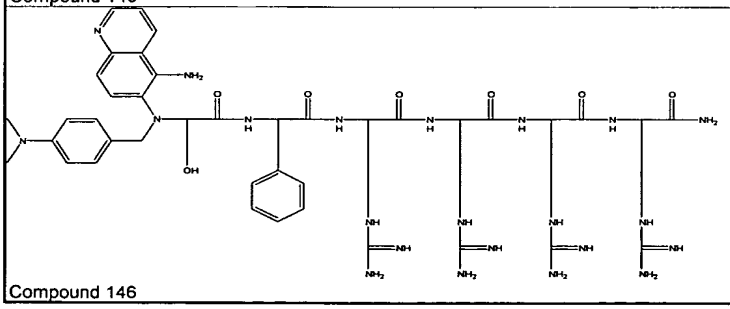
 Compound 117	C53H79N21O7	1122.3436	<10%
 Compound 118	C55H83N21O7	1150.3972	<10%
 Compound 119	C56H85N21O7	1164.424	<10%
 Compound 120	C57H79N21O8	1186.387	<10%
 Compound 121	C59H83N21O9	1230.44	<10%

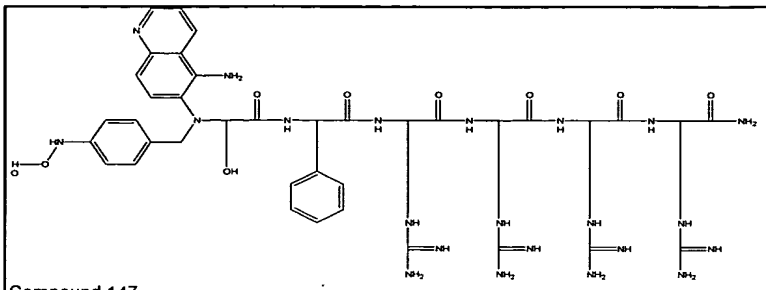
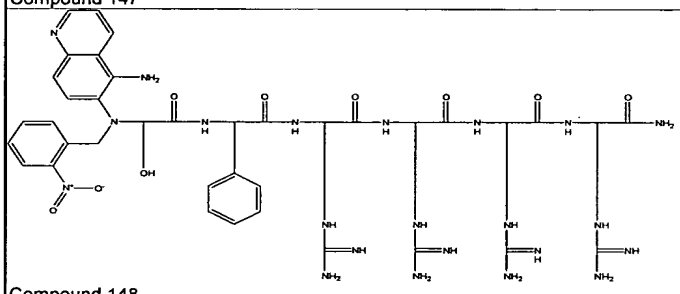
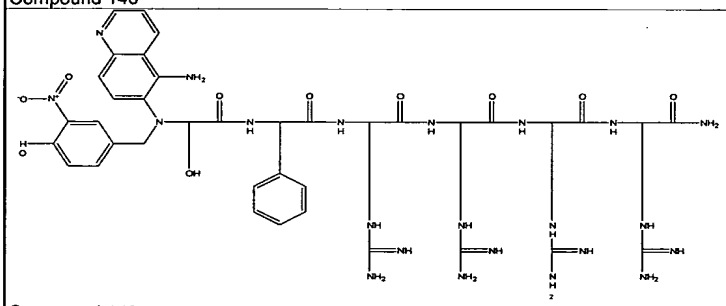
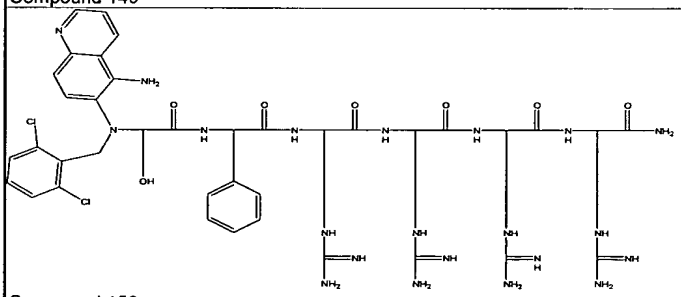
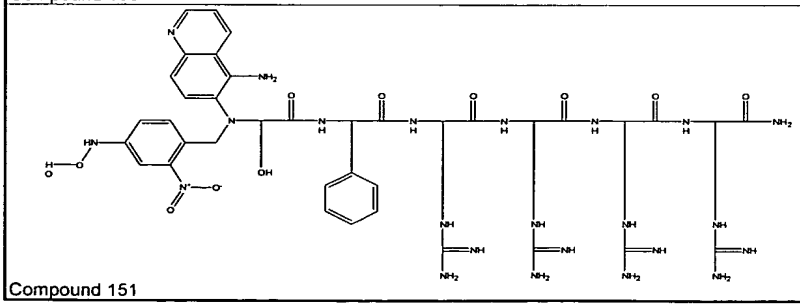
 Compound 122	C59H84N22O8	1229.4552	<10%
 Compound 123	C59H84N22O7	1213.4558	<10%
 Compound 124	C57H80N22O9	1217.401	<10%
 Compound 125	C57H78N22O9	1215.3852	<10%
 Compound 126	C57H78N22O10	1231.3846	<10%

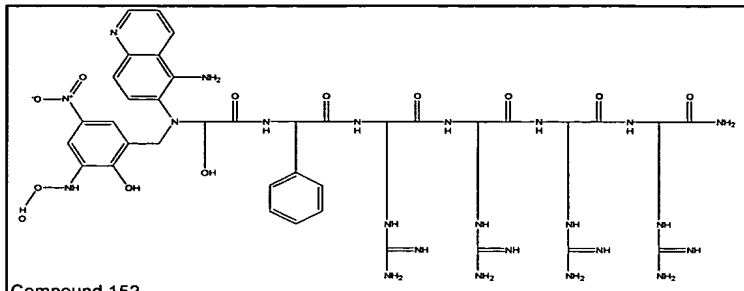
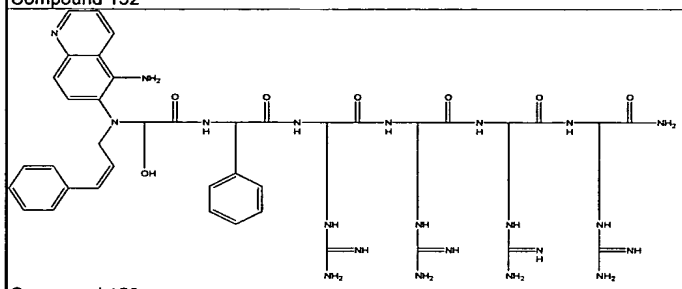
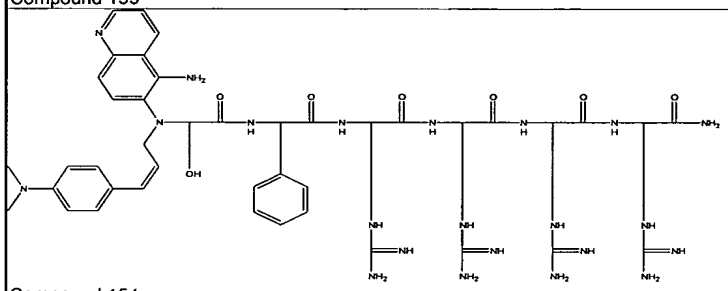
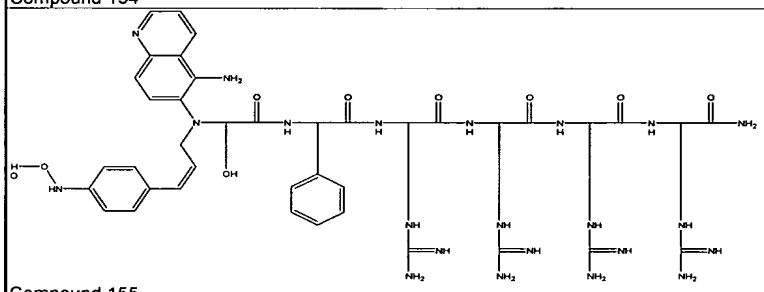
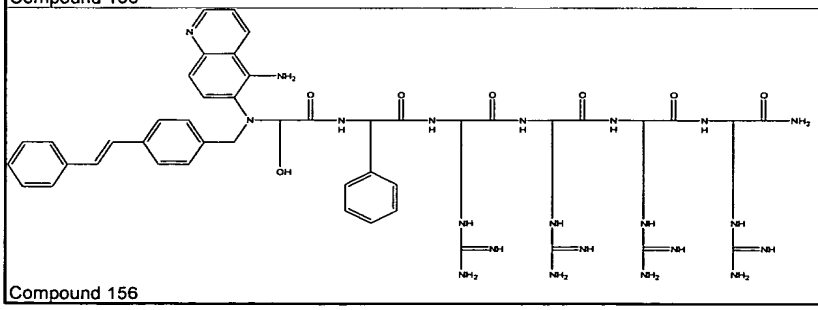
 Compound 127	C57H77Cl2N21O7	1239.2778	<10%
 Compound 128	C57H79N23O11	1262.3986	<10%
 Compound 129	C57H79N23O12	1278.398	<10%
 Compound 130	C59H81N21O7	1196.4254	<10%
 Compound 131	C61H86N22O7	1239.4936	<10%

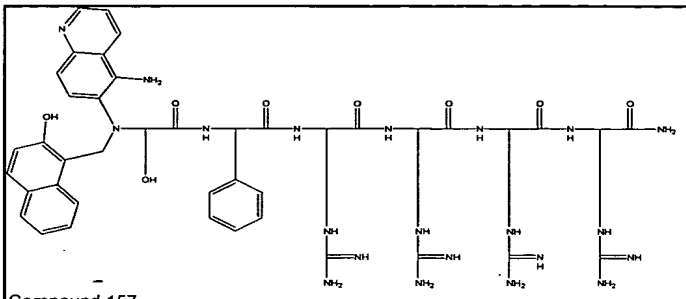
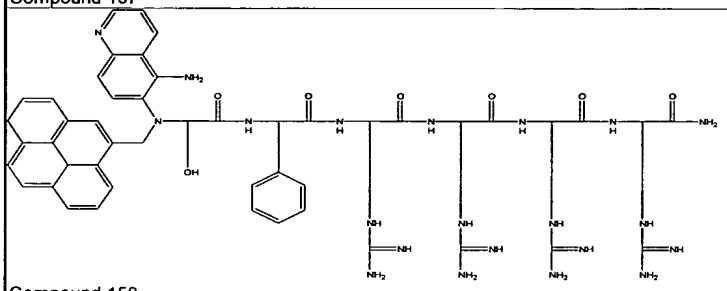
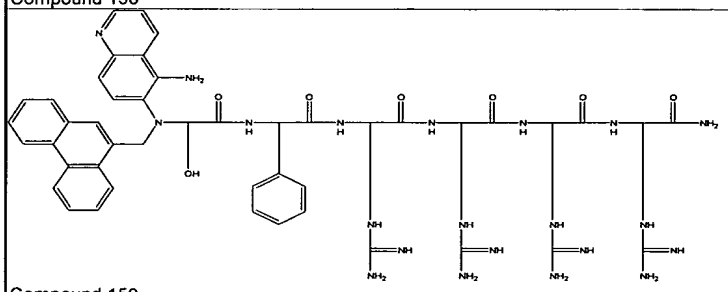
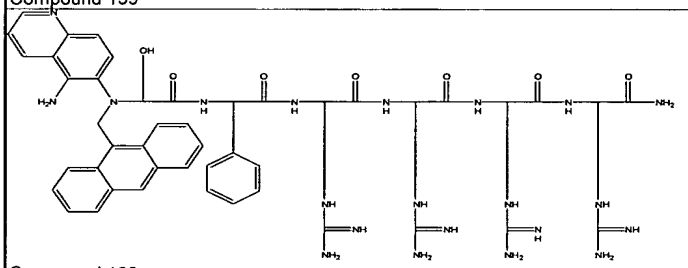
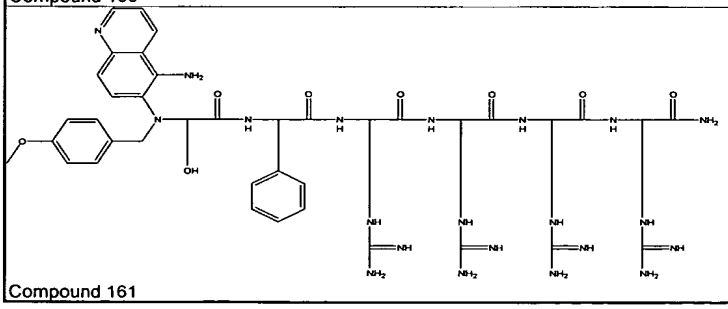
 Compound 132	C59H82N22O9	1243.4388	<10%
 Compound 133	C65H85N21O7	1272.523	<10%
 Compound 134	C61H81N21O8	1236.4468	<10%
 Compound 135	C67H85N21O7	1296.545	<10%
 Compound 136	C65H83N21O7	1270.5072	<10%

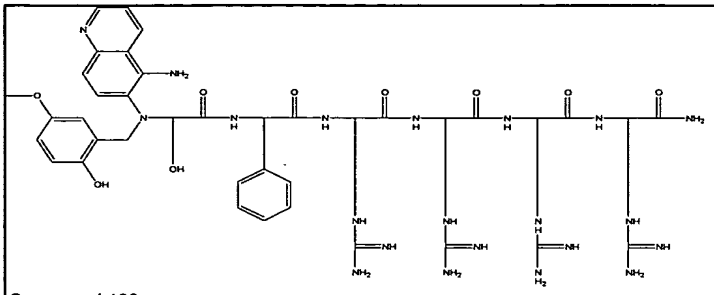
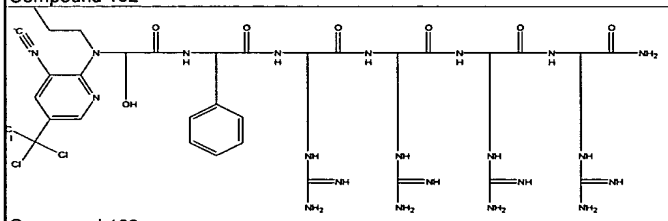
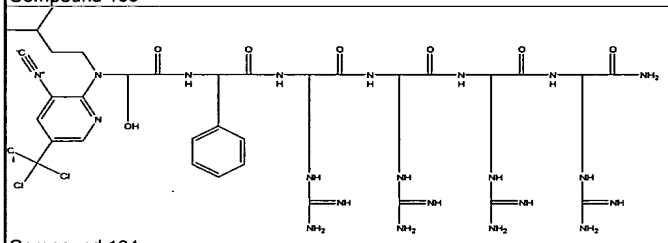
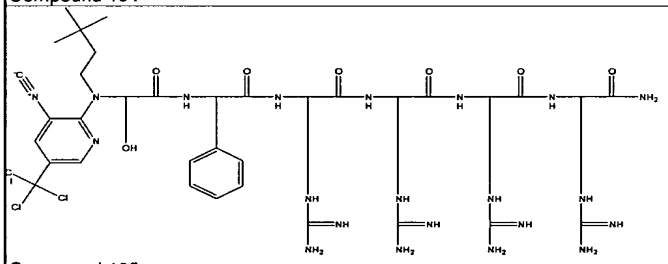
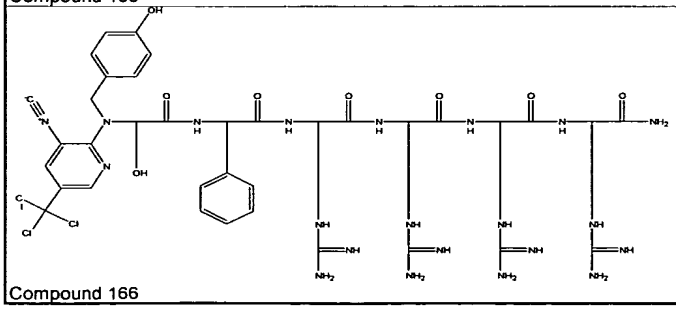
 Compound 137	C65H83N21O7	1270.5072	<10%
 Compound 138	C58H81N21O8	1200.4138	<10%
 Compound 139	C58H81N21O9	1216.4132	<10%
 Compound 140	C48H77N21O7	1060.2728	<10%
 Compound 141	C50H81N21O7	1088.3264	<10%

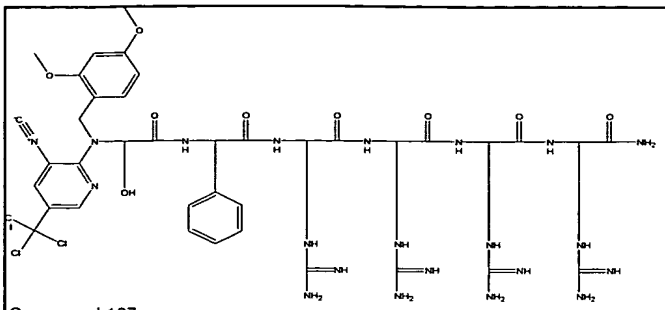
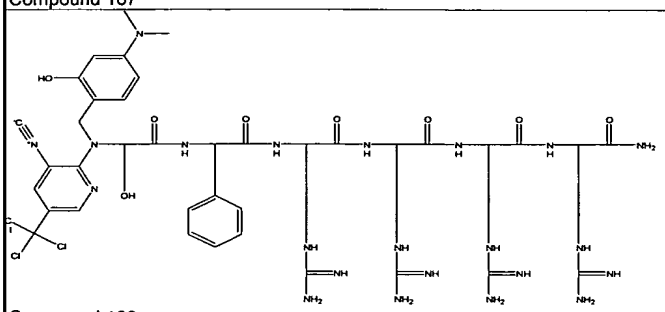
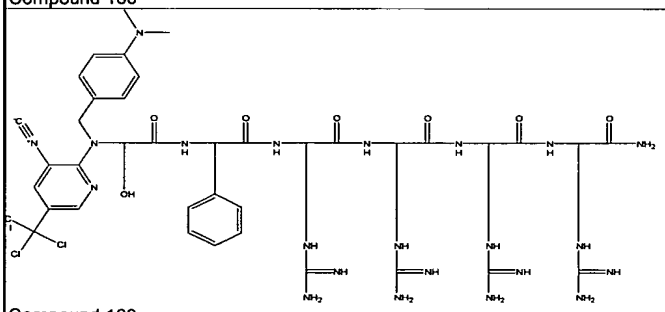
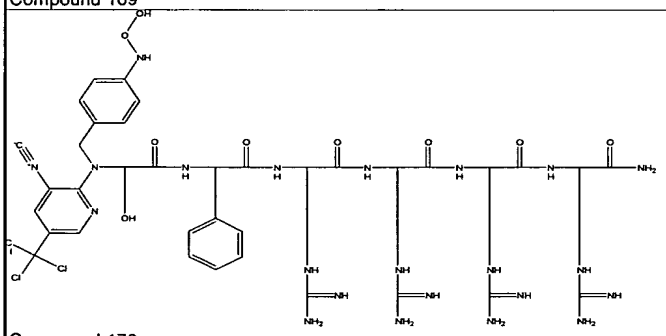
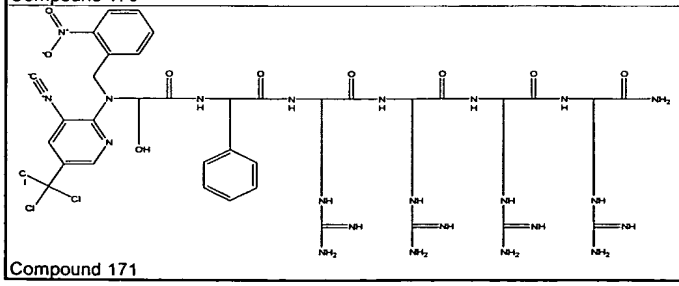
 Compound 142	C51H83N21O7	1102.3532	<10%
 Compound 143	C52H77N21O8	1124.3162	<10%
 Compound 144	C54H81N21O9	1168.3692	<10%
 Compound 145	C54H82N22O8	1167.3844	<10%
 Compound 146	C54H82N22O7	1151.385	<10%

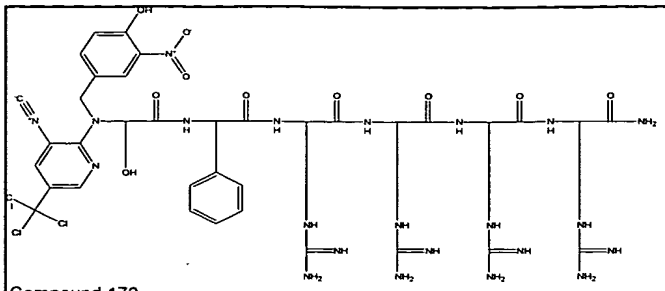
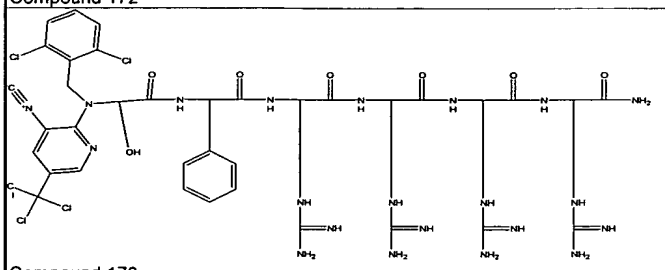
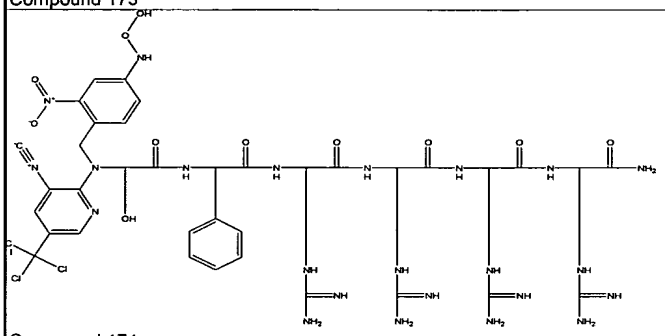
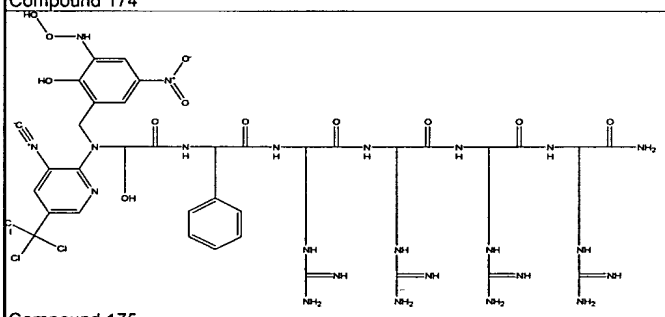
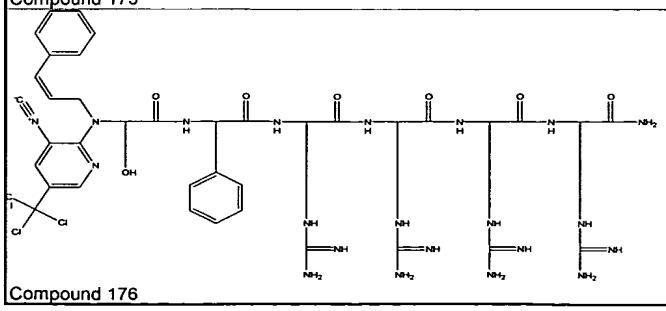
 Compound 147	C52H78N22O9	1155.3302	<10%
 Compound 148	C52H76N22O9	1153.3144	<10%
 Compound 149	C52H76N22O10	1169.3138	<10%
 Compound 150	C52H75Cl2N21O7	1177.207	<10%
 Compound 151	C52H77N23O11	1200.3278	<10%

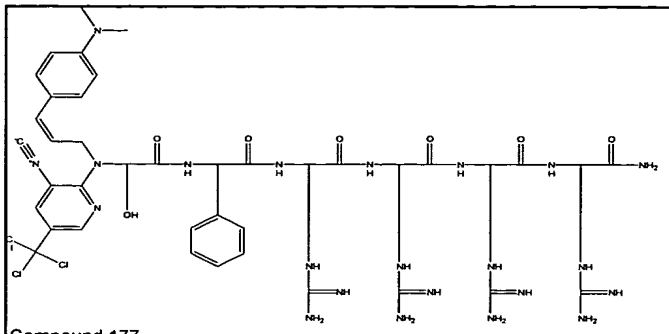
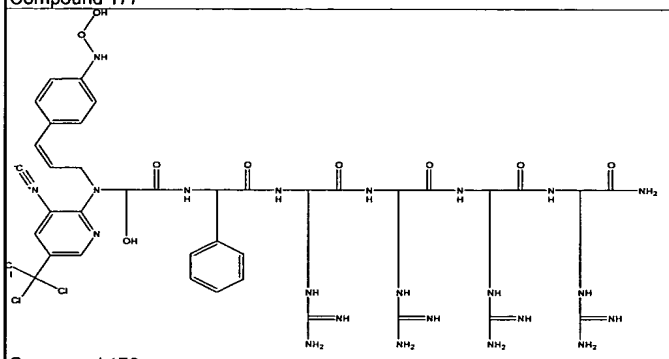
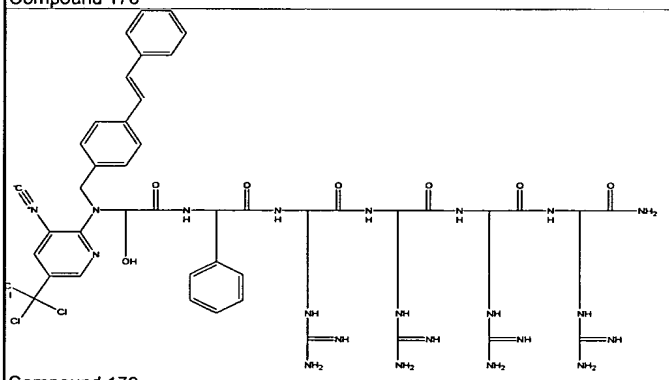
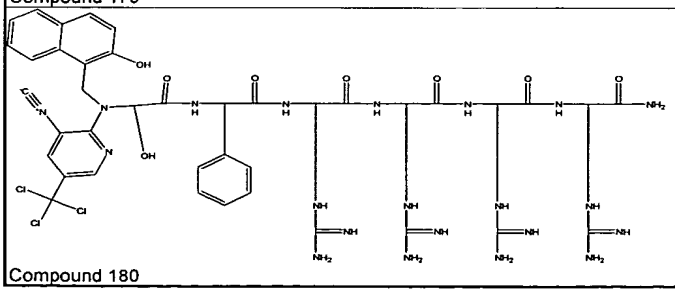
 Compound 152	C52H77N23O12	1216.3272	<10%
 Compound 153	C54H79N21O7	1134.3546	<10%
 Compound 154	C56H84N22O7	1177.4228	<10%
 Compound 155	C54H80N22O9	1181.368	<10%
 Compound 156	C60H83N21O7	1210.4522	<10%

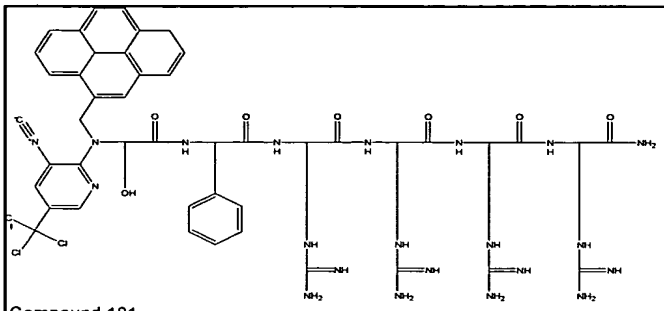
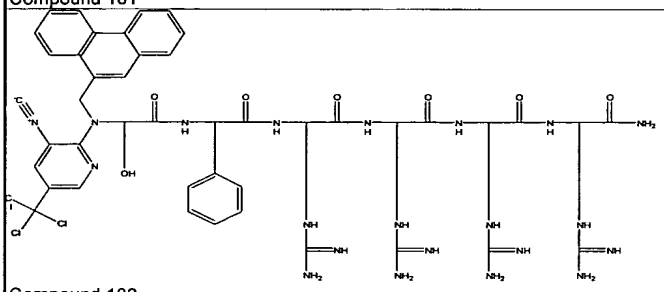
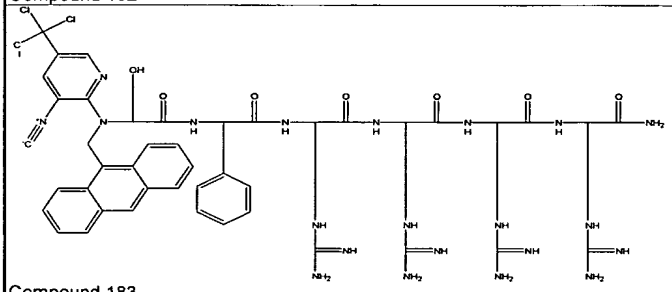
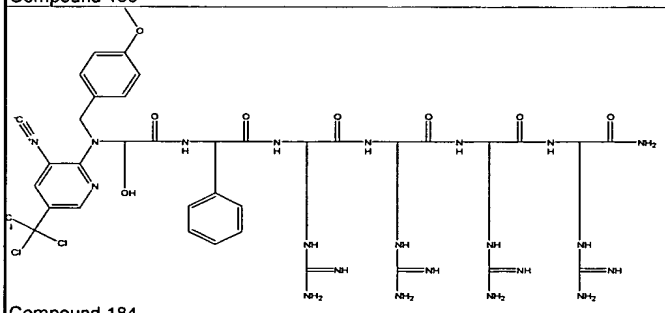
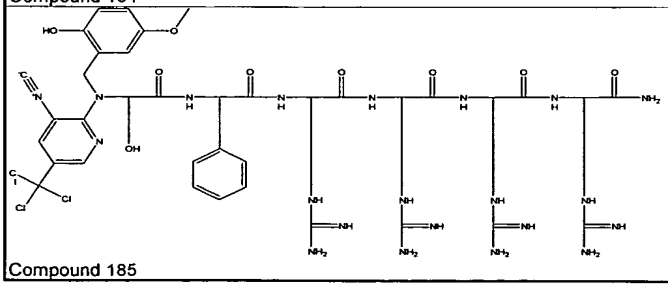
 Compound 157	C56H79N21O8	1174.376	<10%
 Compound 158	C62H83N21O7	1234.4742	<10%
 Compound 159	C60H81N21O7	1208.4364	<10%
 Compound 160	C60H81N21O7	1208.4364	<10%
 Compound 161	C53H79N21O8	1138.343	<10%

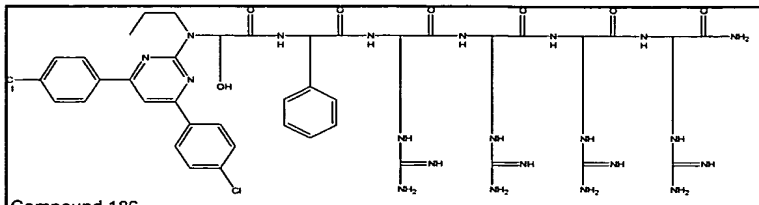
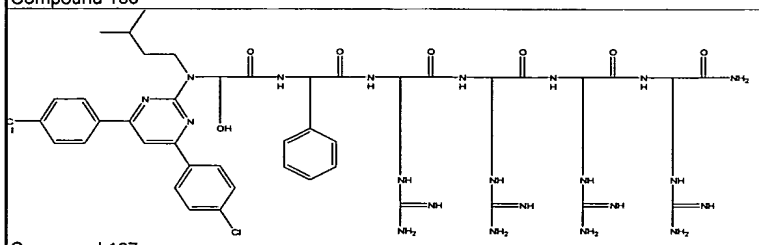
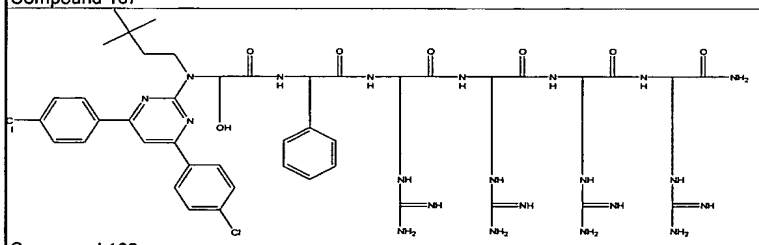
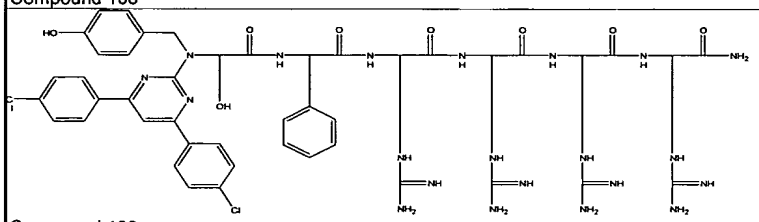
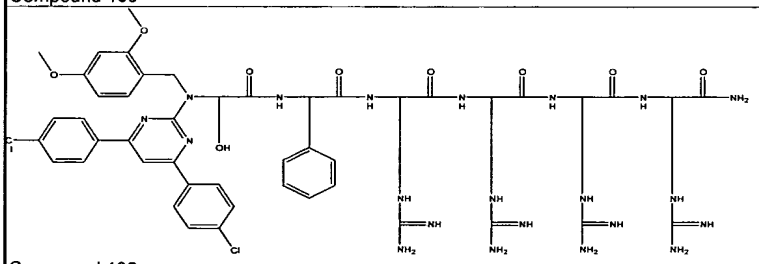
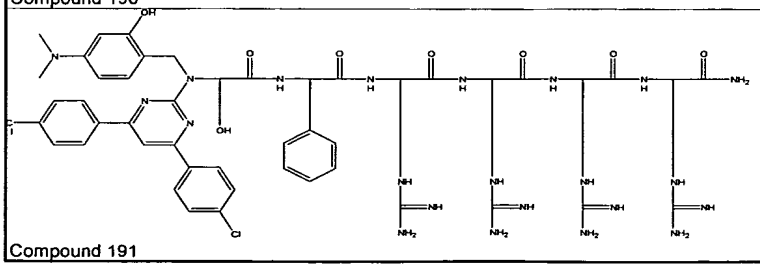
 Compound 162	C53H79N21O9	1154.3424	<10%
 Compound 163	C46H72Cl3N21O7	1137.5703	<10%
 Compound 164	C48H76Cl3N21O7	1165.6239	<10%
 Compound 165	C49H78Cl3N21O7	1179.6507	<10%
 Compound 166	C50H72Cl3N21O8	1201.6137	<10%

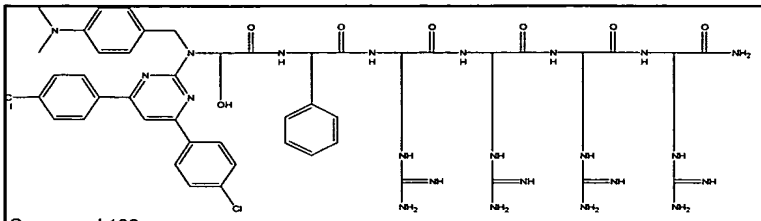
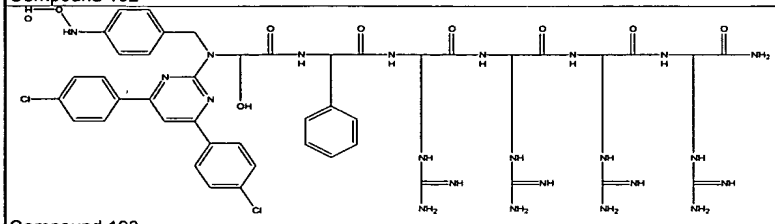
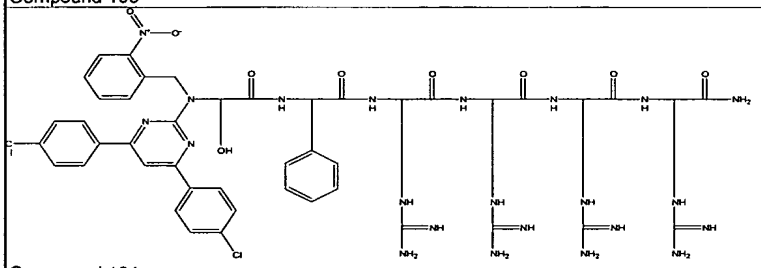
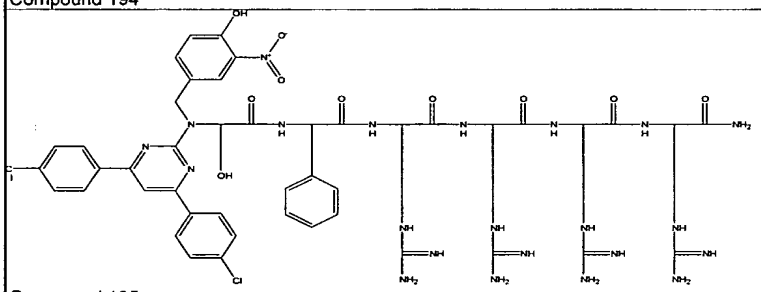
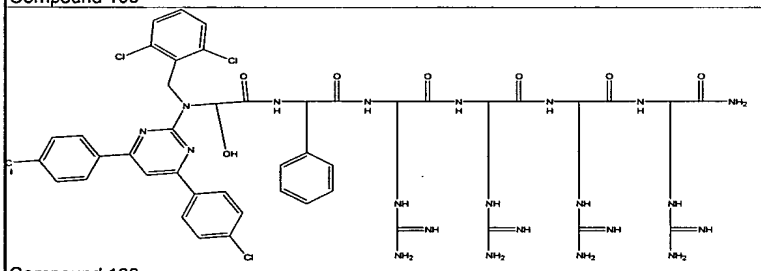
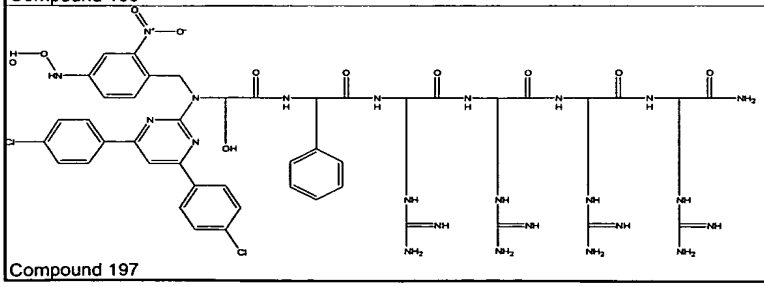
 Compound 167	C52H76Cl3N21O9	1245.6667	<10%
 Compound 168	C52H77Cl3N22O8	1244.6819	<10%
 Compound 169	C52H77Cl3N22O7	1228.6825	<10%
 Compound 170	C50H73Cl3N22O9	1232.6277	<10%
 Compound 171	C50H71Cl3N22O9	1230.6119	<10%

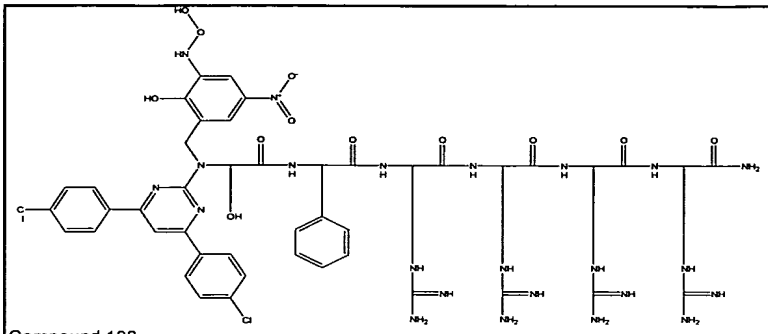
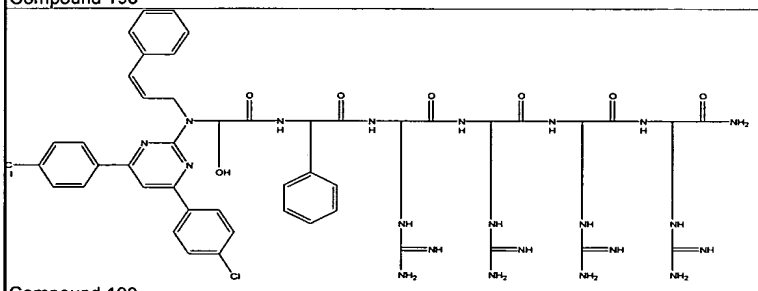
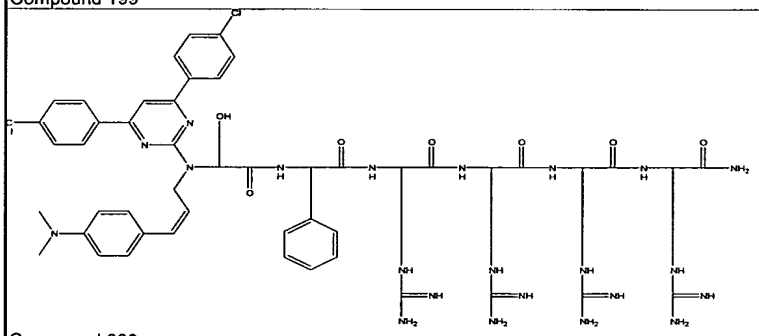
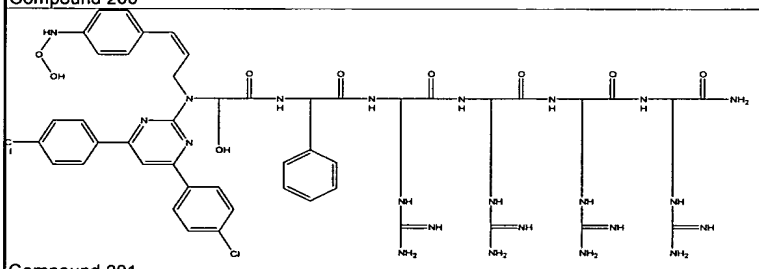
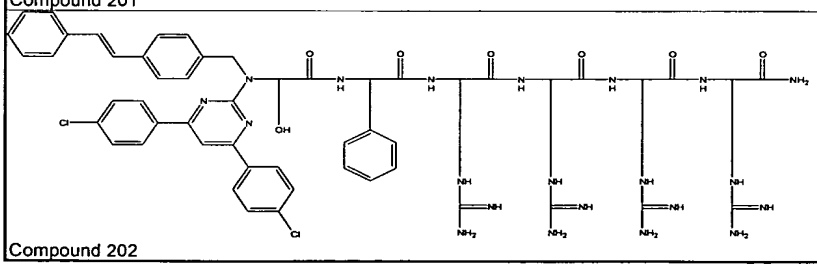
 Compound 172	C50H71Cl3N22O10	1246.6113	<10%
 Compound 173	C50H70Cl5N21O7	1254.5045	<10%
 Compound 174	C50H72Cl3N23O11	1277.6253	<10%
 Compound 175	C50H72Cl3N23O12	1293.6247	<10%
 Compound 176	C52H74Cl3N21O7	1211.6521	<10%

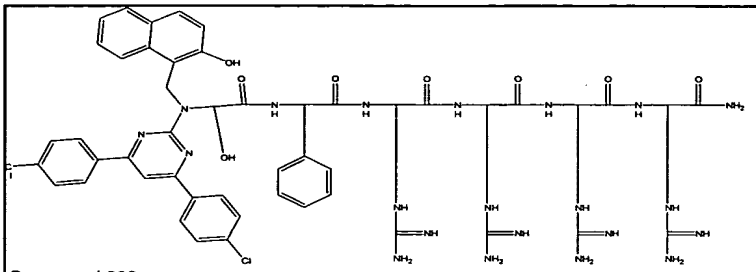
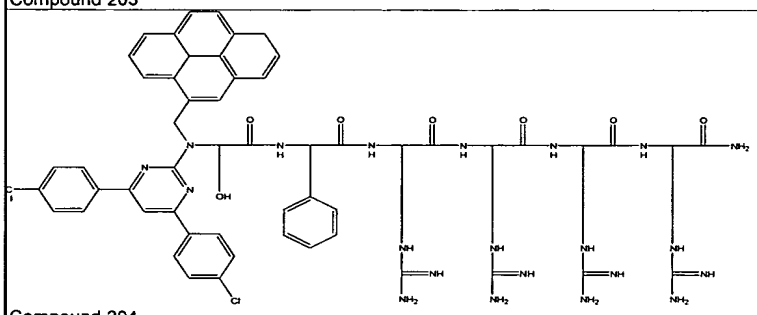
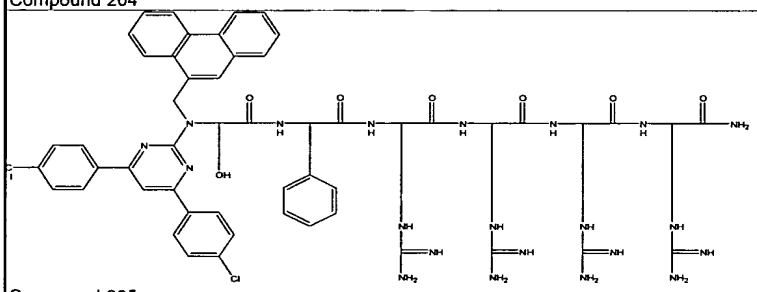
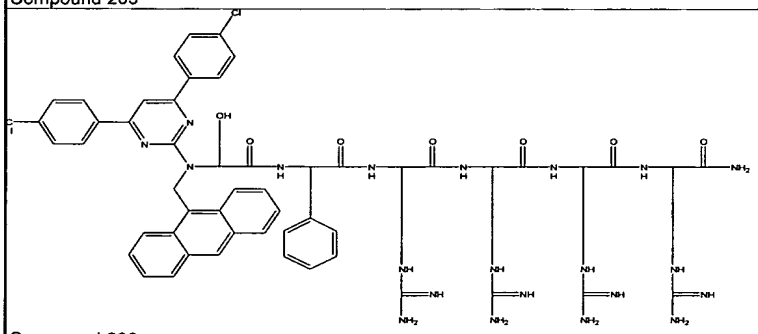
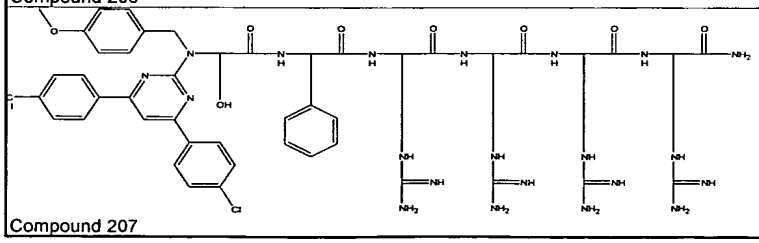
 Compound 177	C54H79Cl3N22O7	1254.7203	<10%
 Compound 178	C52H75Cl3N22O9	1258.6655	<10%
 Compound 179	C58H78Cl3N21O7	1287.7497	<10%
 Compound 180	C54H74Cl3N21O8	1251.6735	<10%

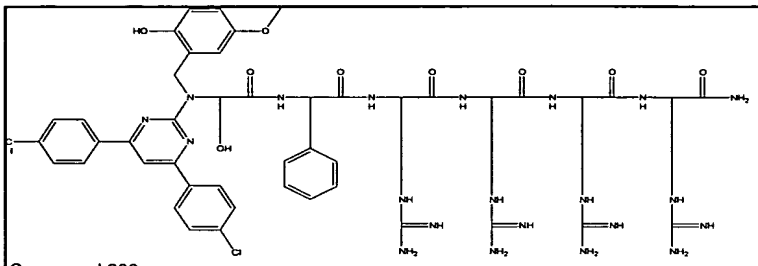
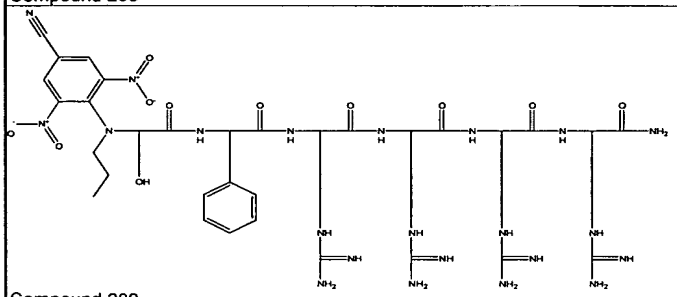
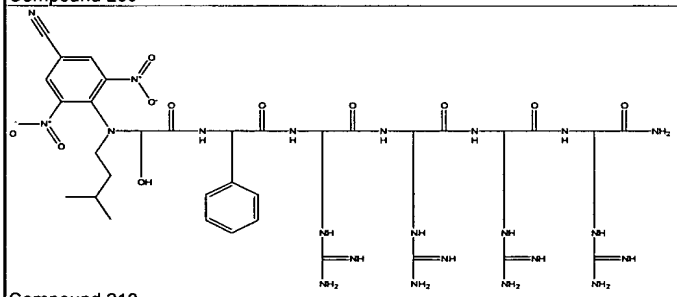
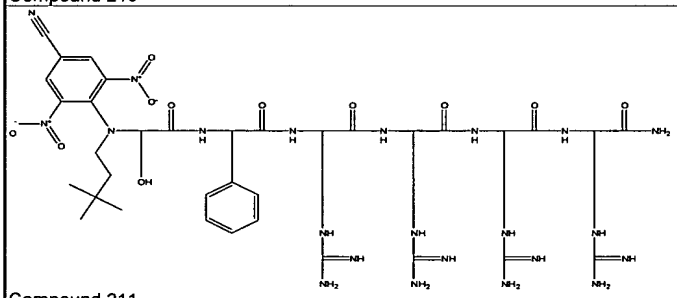
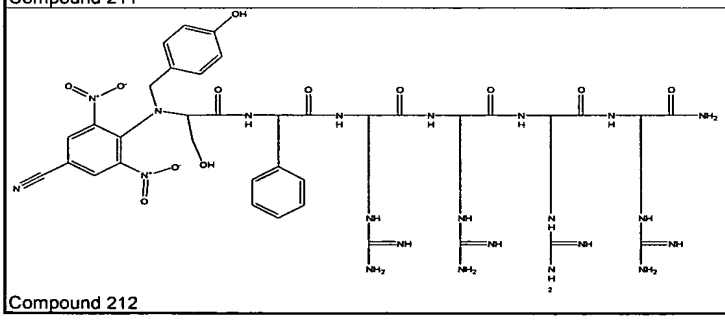
 Compound 181	C60H78Cl3N21O7	1311.7717	<10%
 Compound 182	C58H76Cl3N21O7	1285.7339	<10%
 Compound 183	C58H76Cl3N21O7	1285.7339	<10%
 Compound 184	C51H74Cl3N21O8	1215.6405	<10%
 Compound 185	C51H74Cl3N21O9	1231.6399	<10%

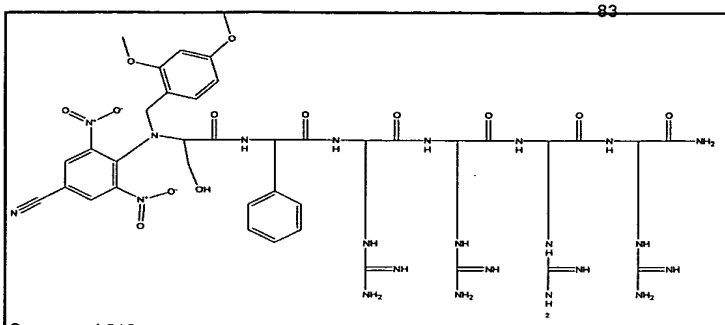
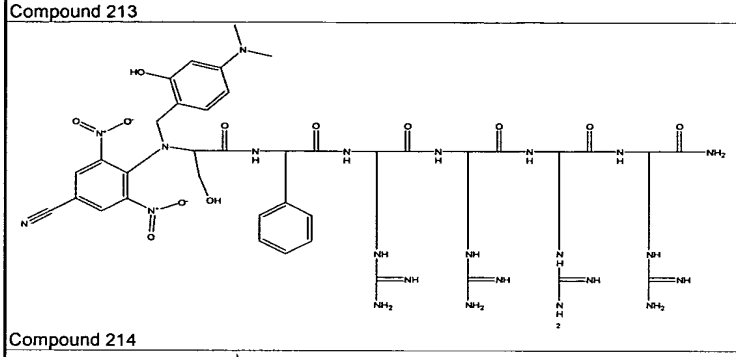
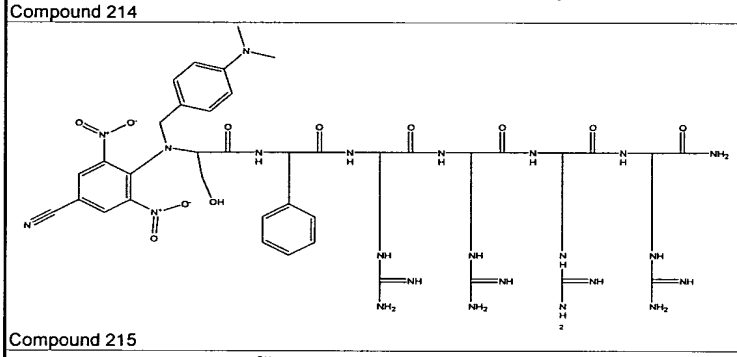
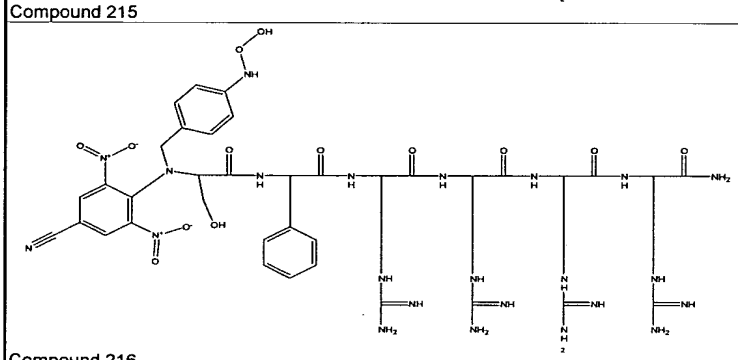
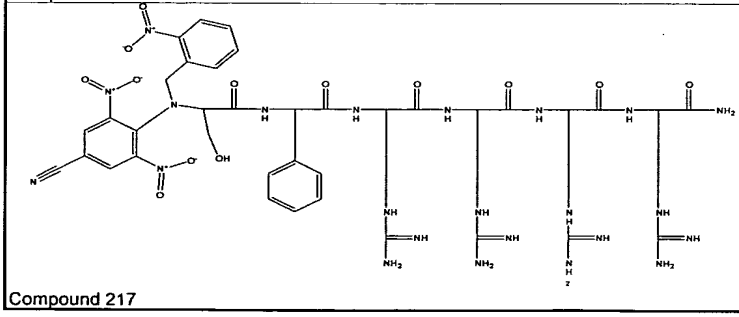
 Compound 186	C55H79Cl2N21O7	1217.2716	<10%
 Compound 187	C57H83Cl2N21O7	1245.3252	<10%
 Compound 188	C58H85Cl2N21O7	1259.352	<10%
 Compound 189	C59H79Cl2N21O8	1281.315	<10%
 Compound 190	C61H83Cl2N21O9	1325.368	<10%
 Compound 191	C61H84Cl2N22O8	1324.3832	<10%

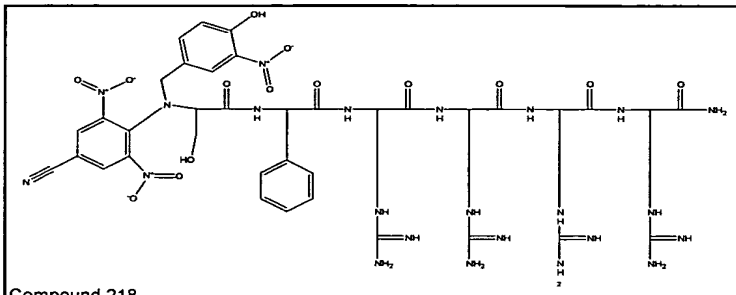
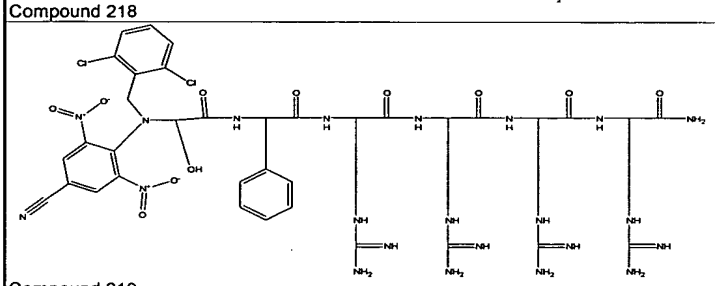
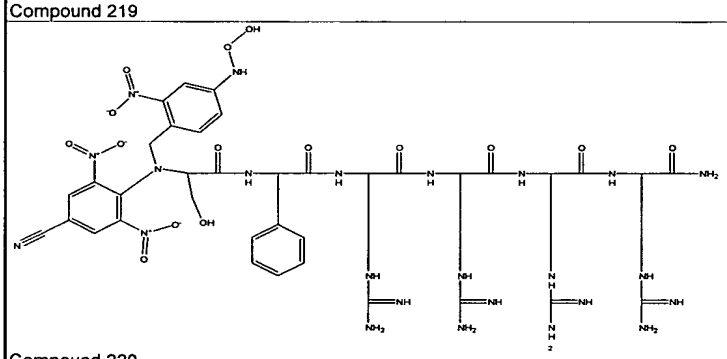
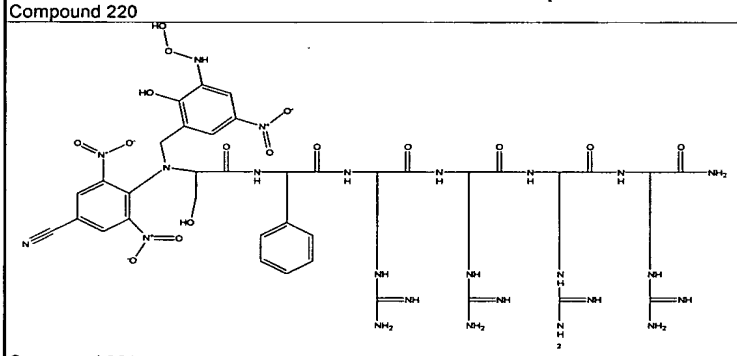
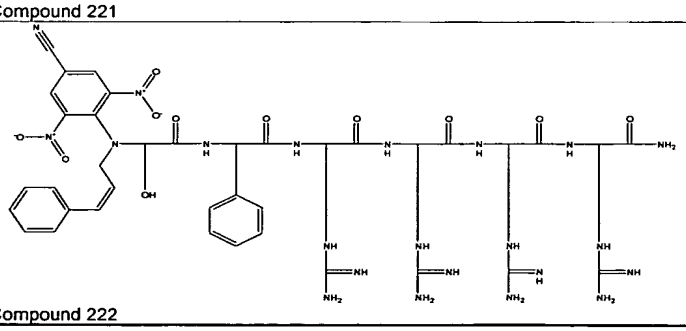
 Compound 192	C61H84Cl2N22O7	1308.3838	<10%
 Compound 193	C59H80Cl2N22O9	1312.329	<10%
 Compound 194	C59H78Cl2N22O9	1310.3132	<10%
 Compound 195	C59H78Cl2N22O10	1326.3126	<10%
 Compound 196	C59H77Cl4N21O7	1334.2058	<10%
 Compound 197	C59H79Cl2N23O11	1357.3266	<10%

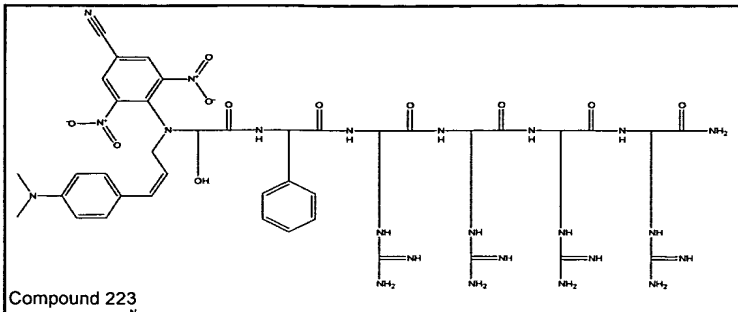
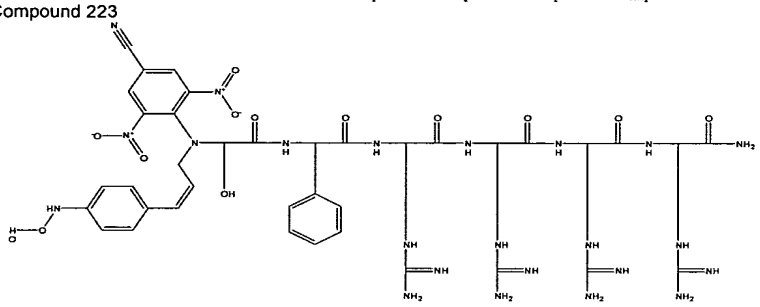
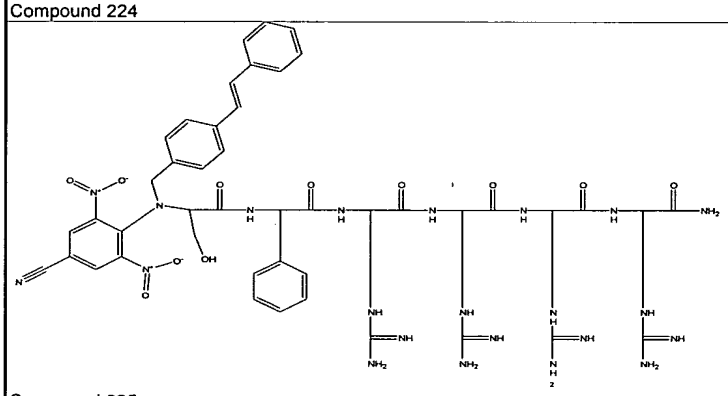
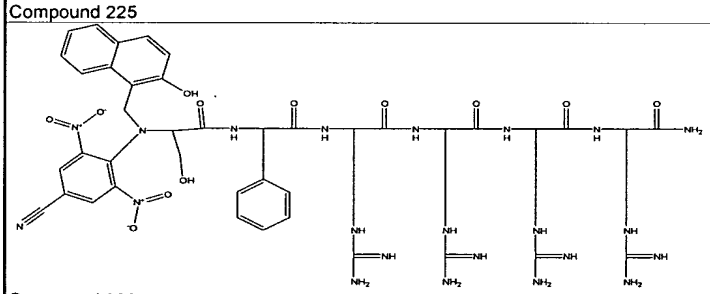
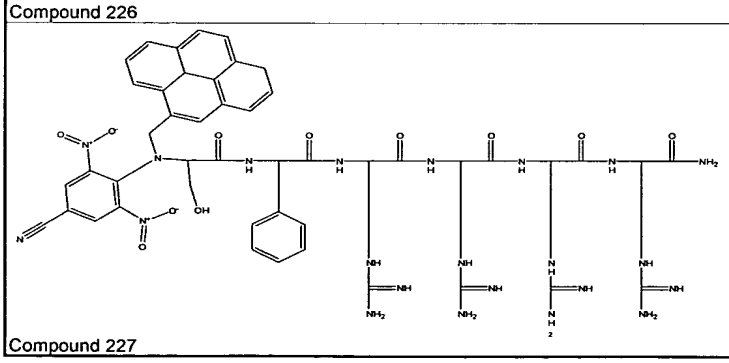
 Compound 198	C59H79Cl2N23O12	1373.326	<10%
 Compound 199	C61H81Cl2N21O7	1291.3534	<10%
 Compound 200	C63H86Cl2N22O7	1334.4216	<10%
 Compound 201	C61H82Cl2N22O9	1338.3668	<10%
 Compound 202	C67H85Cl2N21O7	1367.451	<10%

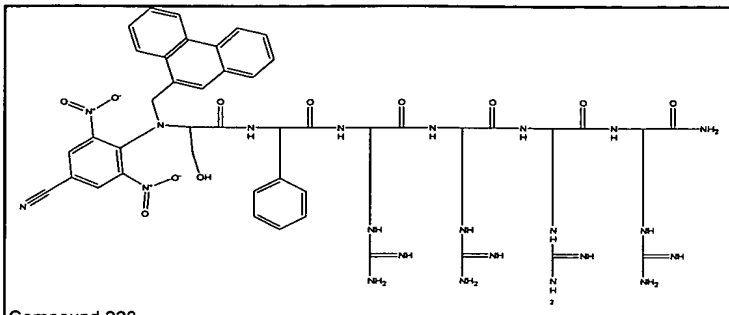
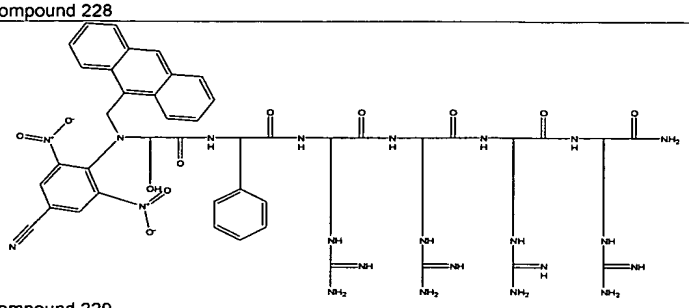
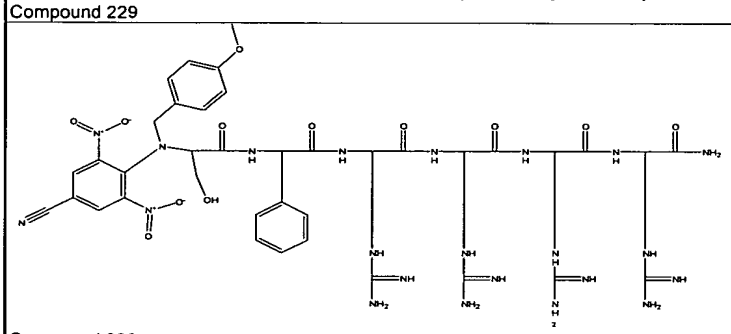
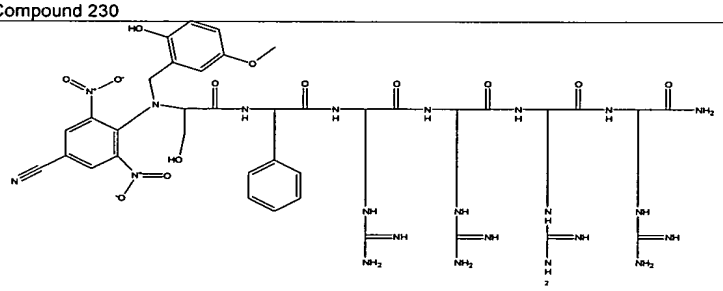
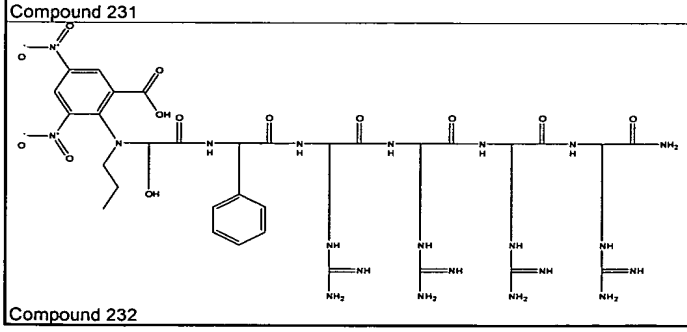
 Compound 203	C63H81Cl2N21O8	1331.3748	<10%
 Compound 204	C69H85Cl2N21O7	1391.473	<10%
 Compound 205	C67H83Cl2N21O7	1365.4352	<10%
 Compound 206	C67H83Cl2N21O7	1365.4352	<10%
 Compound 207	C60H81Cl2N21O8	1295.3418	<10%

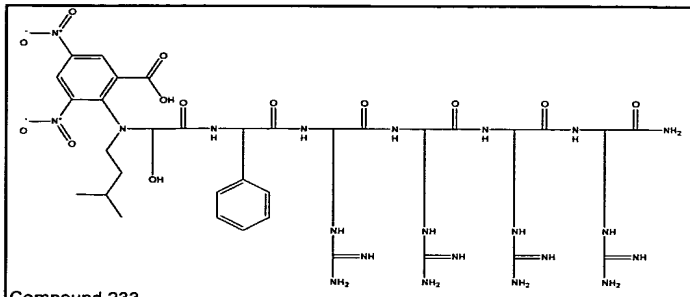
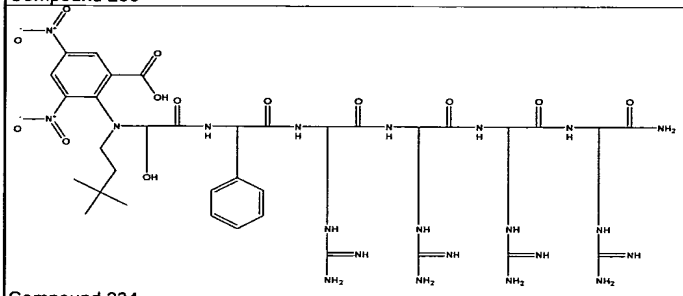
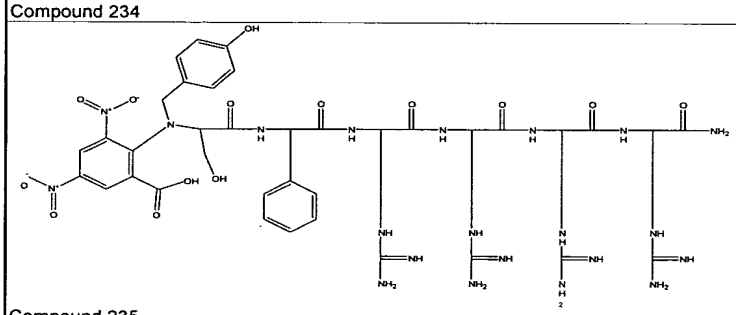
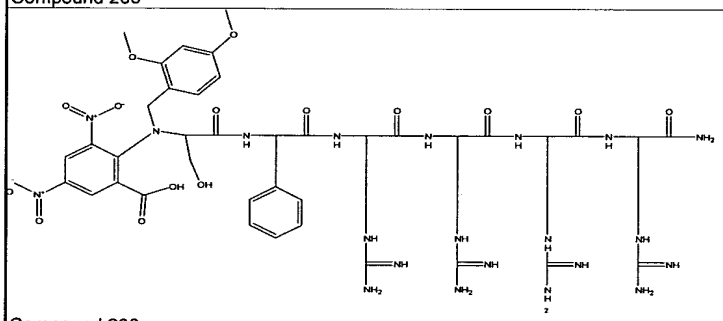
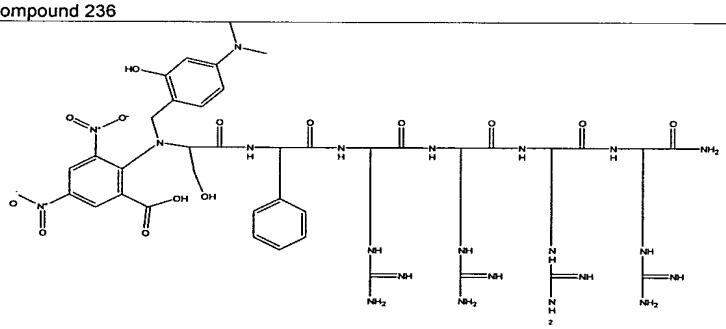
 Compound 208	C60H81Cl2N21O9	1311.3412	<10%
 Compound 209	C46H72N22O11	1109.2156	<10%
 Compound 210	C48H76N22O11	1137.2692	<10%
 Compound 211	C49H78N22O11	1151.296	<10%
 Compound 212	C50H72N22O12	1173.259	<10%

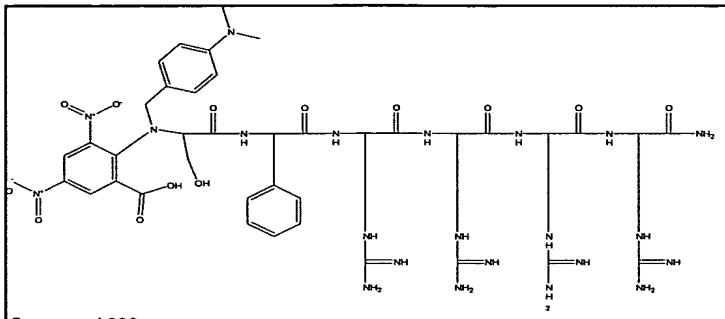
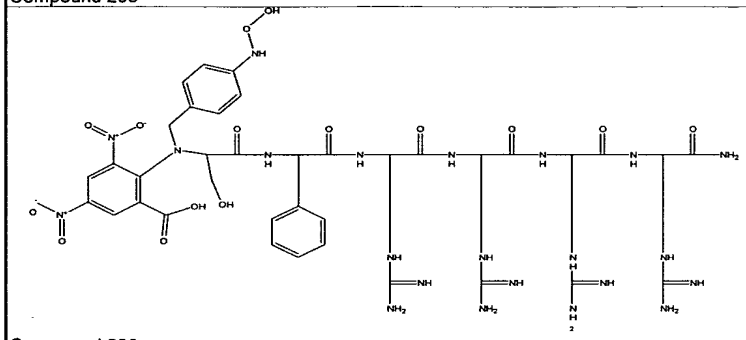
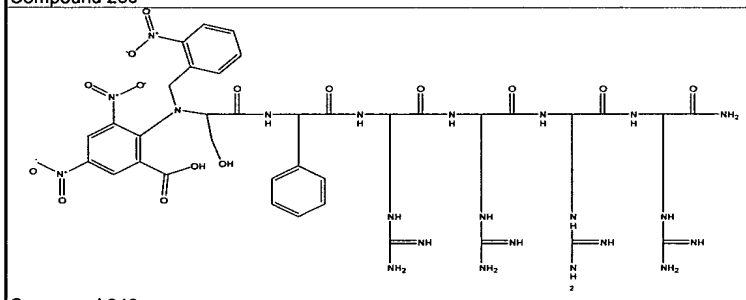
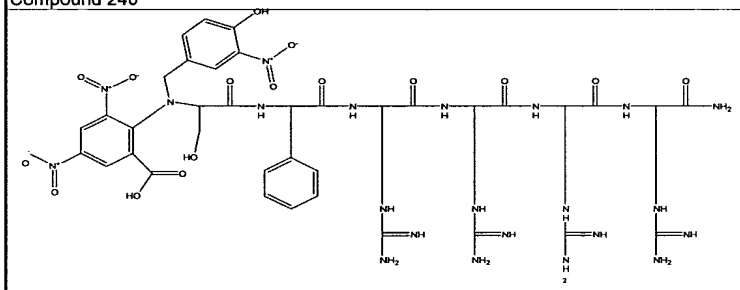
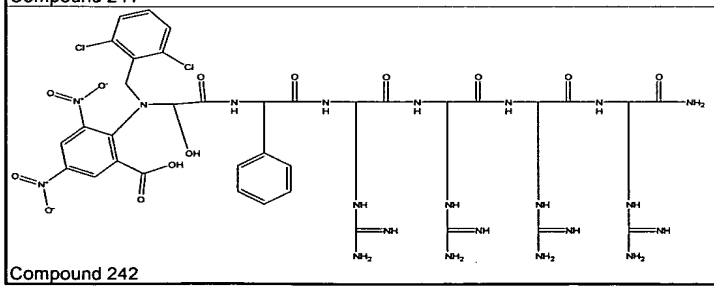
 Compound 213	C52H76N22O13	1217.312	<10%
 Compound 214	C52H77N23O12	1216.3272	<10%
 Compound 215	C52H77N23O11	1200.3278	<10%
 Compound 216	C50H73N23O13	1204.273	<10%
 Compound 217	C50H71N23O13	1202.2572	<10%

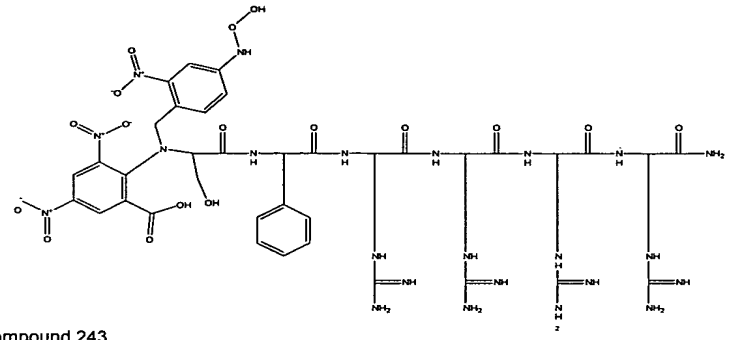
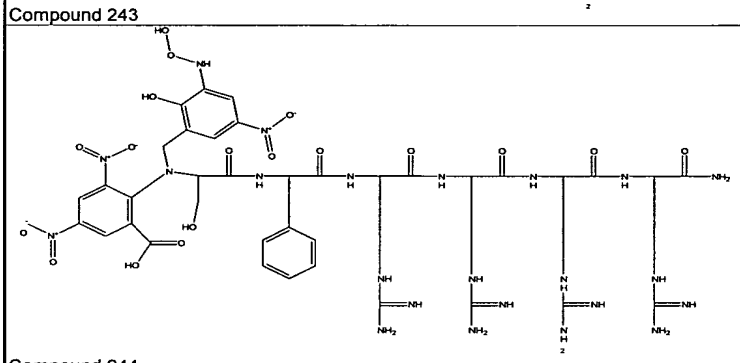
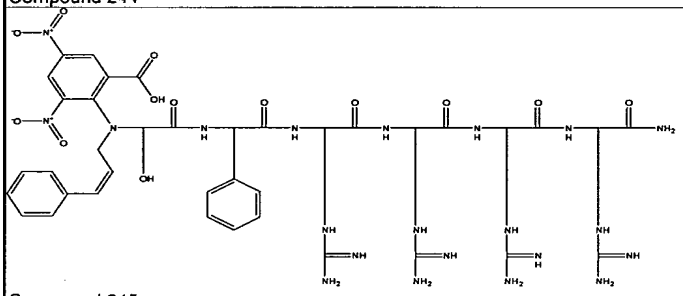
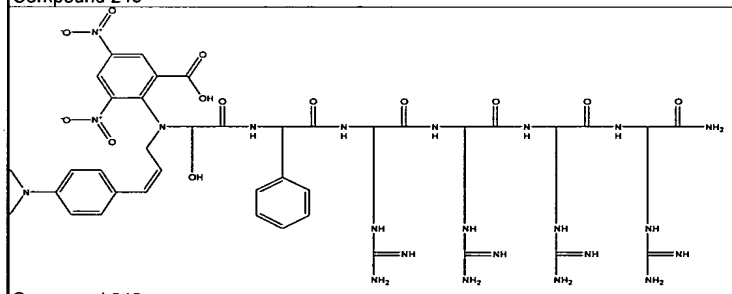
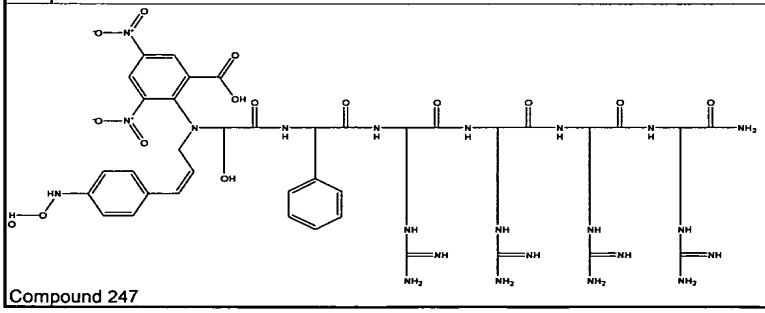
 Compound 218	C50H71N23O14	1218.2566	<10%
 Compound 219	C50H70Cl2N22O11	1226.1498	<10%
 Compound 220	C50H72N24O15	1249.2706	<10%
 Compound 221	C50H72N24O16	1265.27	<10%
 Compound 222	C52H74N22O11	1183.2974	<10%

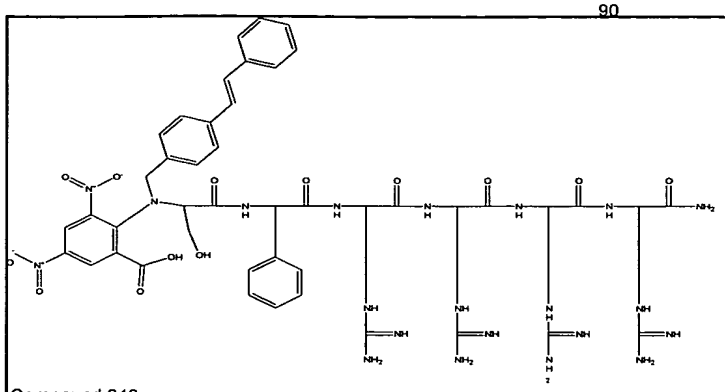
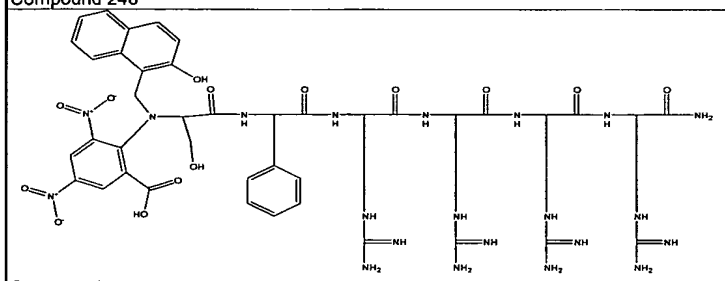
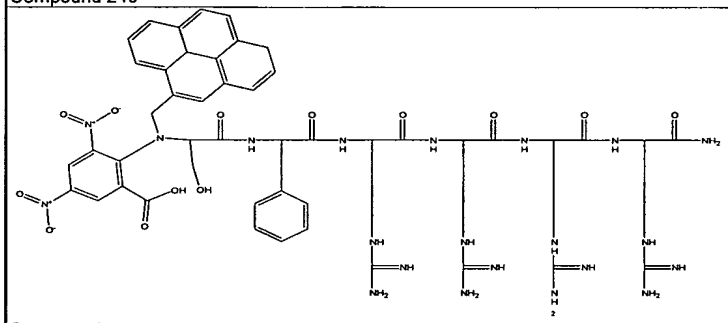
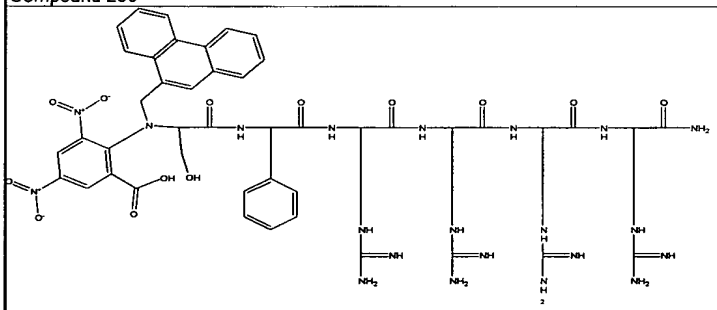
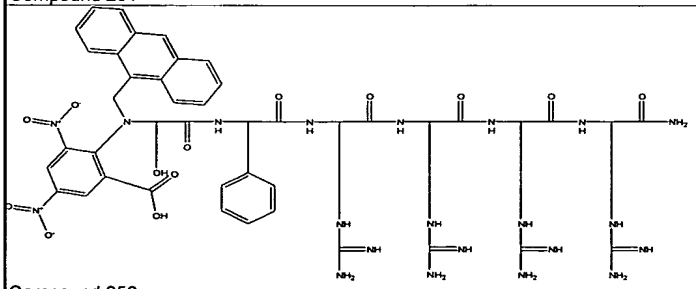
 Compound 223	C54H79N23O11	1226.3656	<10%
 Compound 224	C52H75N23O13	1230.3108	<10%
 Compound 225	C58H78N22O11	1259.395	<10%
 Compound 226	C54H74N22O12	1223.3188	<10%
 Compound 227	C60H78N22O11	1283.417	<10%

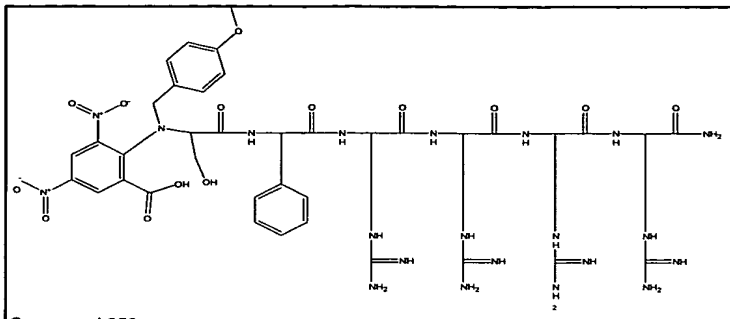
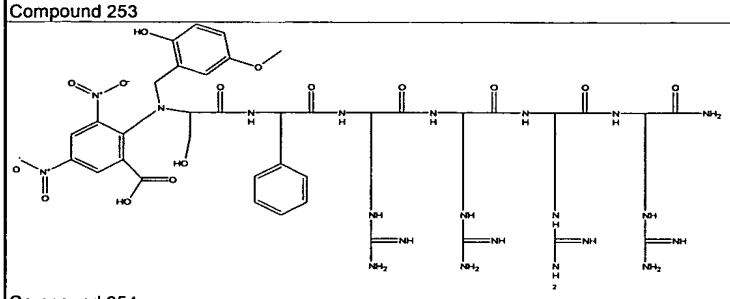
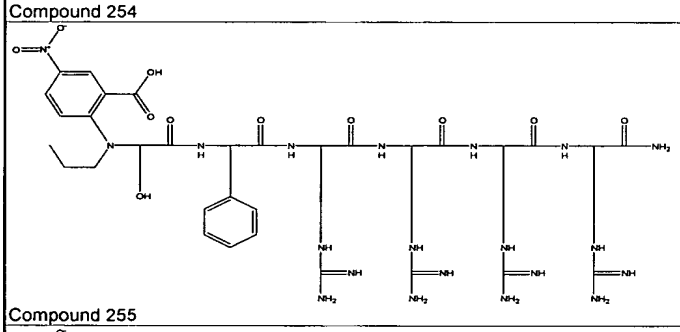
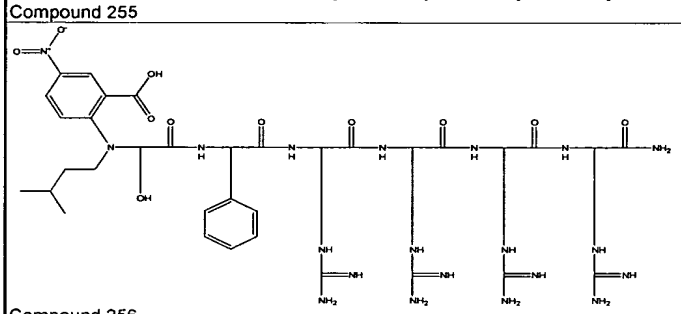
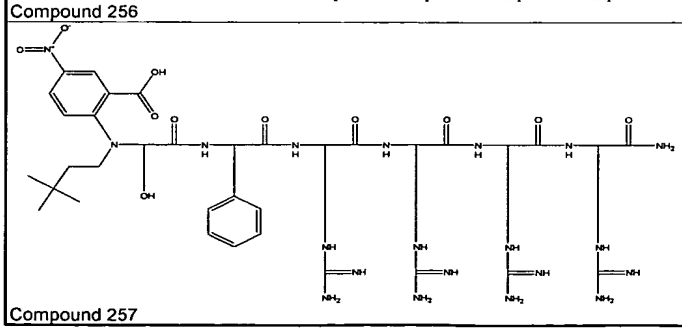
 Compound 228	C58H76N22O11	1257.3792	<10%
 Compound 229	C58H76N22O11	1257.3792	<10%
 Compound 230	C51H74N22O12	1187.2858	<10%
 Compound 231	C51H74N22O13	1203.2852	<10%
 Compound 232	C46H73N21O13	1128.2156	<10%

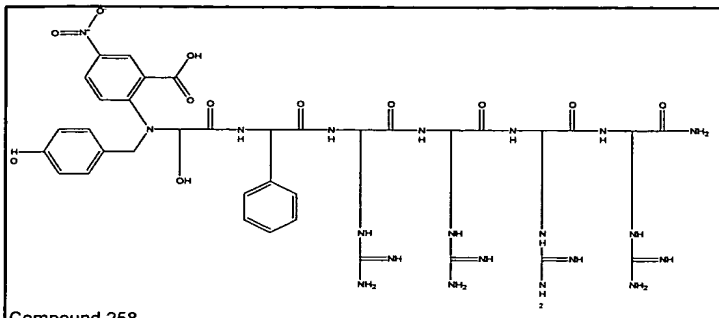
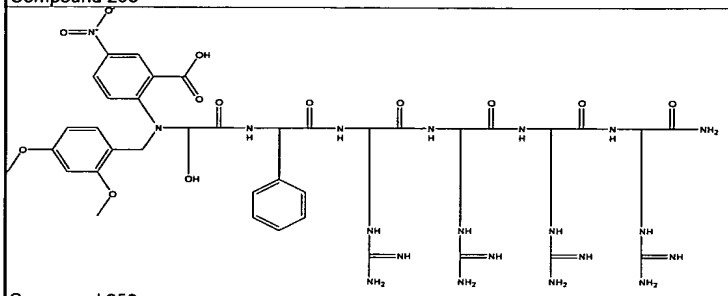
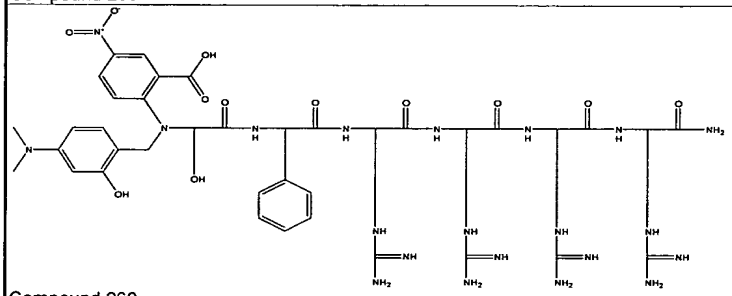
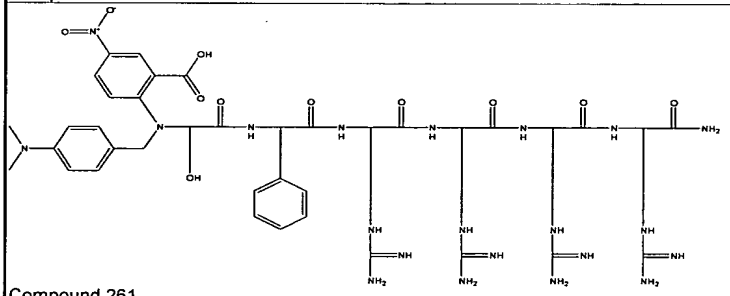
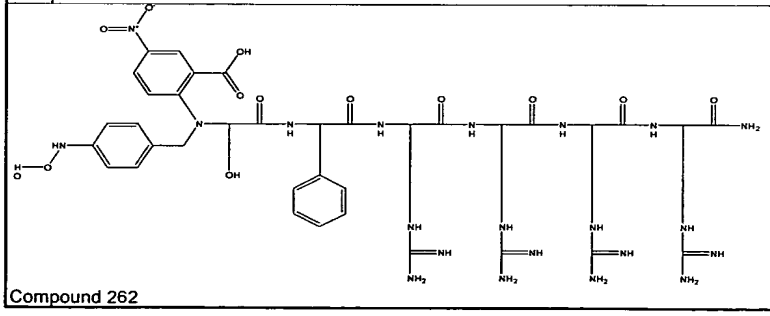
 Compound 233	C48H77N21O13	1156.2692	<10%
 Compound 234	C49H79N21O13	1170.296	<10%
 Compound 235	C50H73N21O14	1192.259	<10%
 Compound 236	C52H77N21O15	1236.312	<10%
 Compound 237	C52H78N22O14	1235.3272	<10%

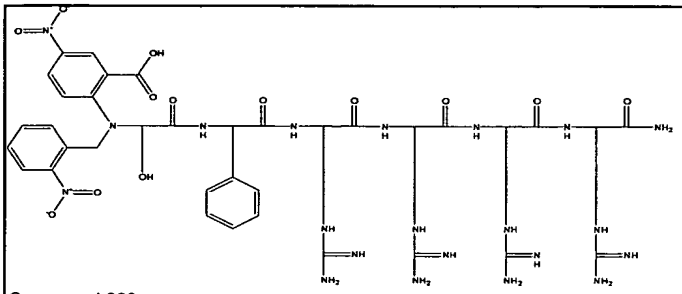
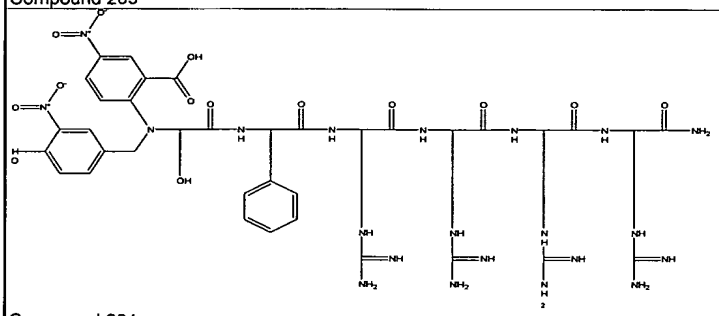
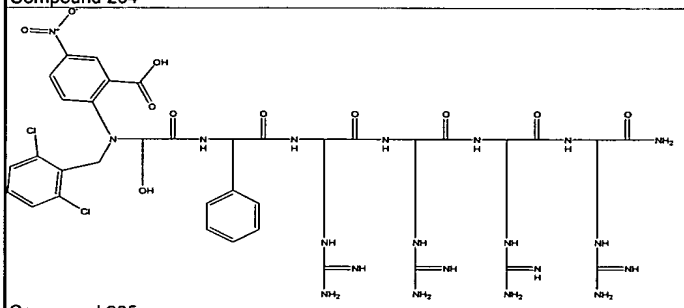
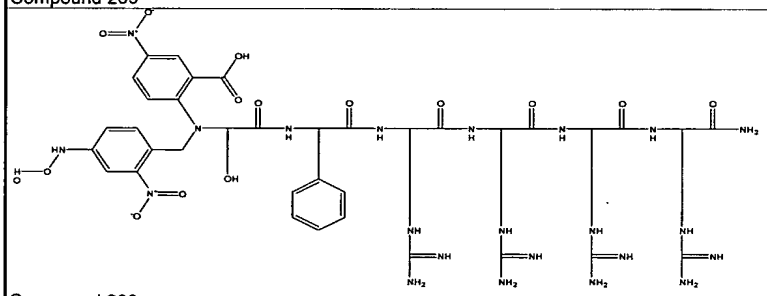
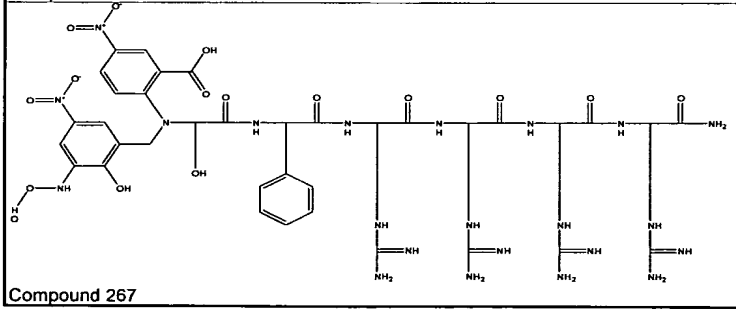
 Compound 238	C52H78N22O13	1219.3278	<10%
 Compound 239	C50H74N22O15	1223.273	<10%
 Compound 240	C50H72N22O15	1221.2572	<10%
 Compound 241	C50H72N22O16	1237.2566	<10%
 Compound 242	C50H71Cl2N21O13	1245.1498	<10%

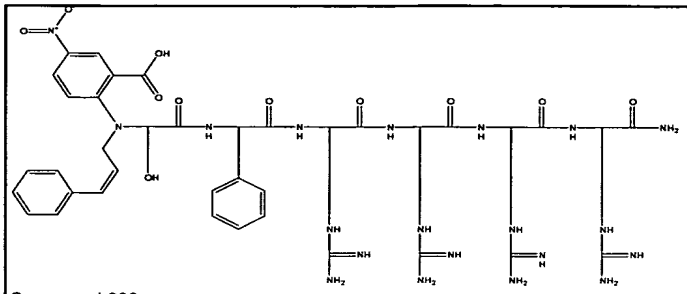
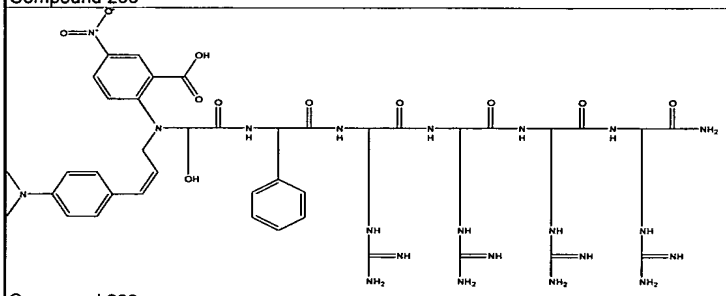
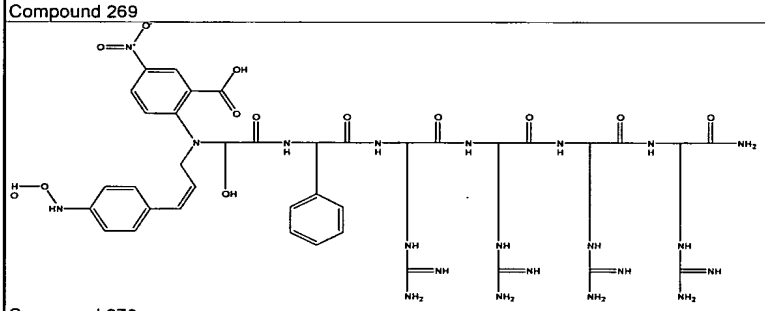
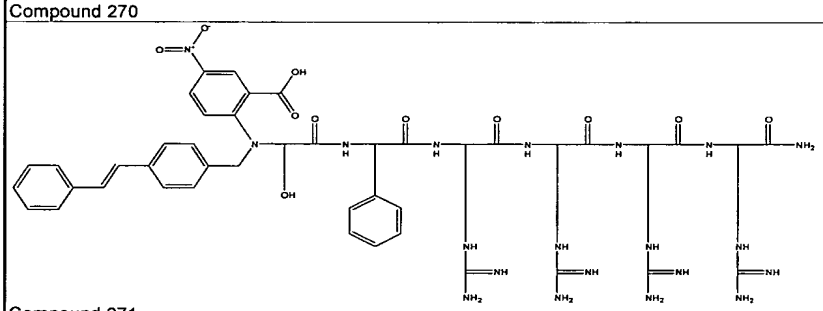
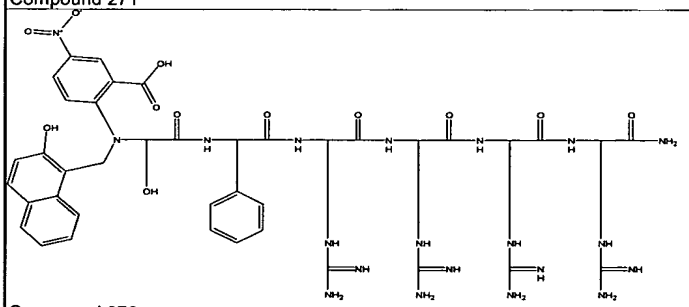
 Compound 243	C50H73N23O17	1268.2706	<10%
 Compound 244	C50H73N23O18	1284.27	<10%
 Compound 245	C52H75N21O13	1202.2974	<10%
 Compound 246	C54H80N22O13	1245.3656	<10%
 Compound 247	C52H76N22O15	1249.3108	<10%

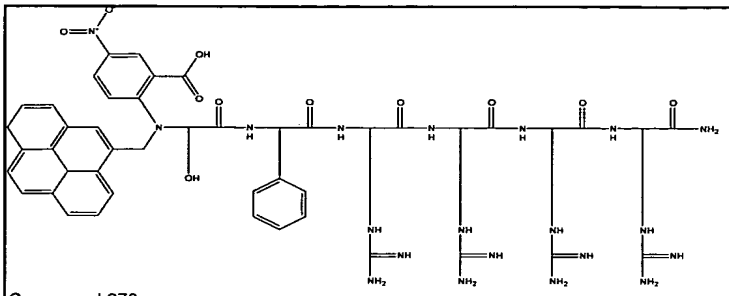
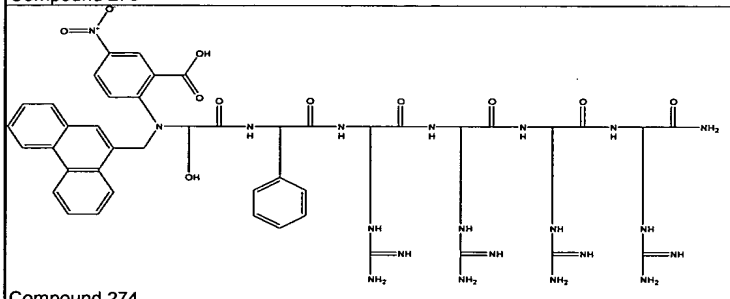
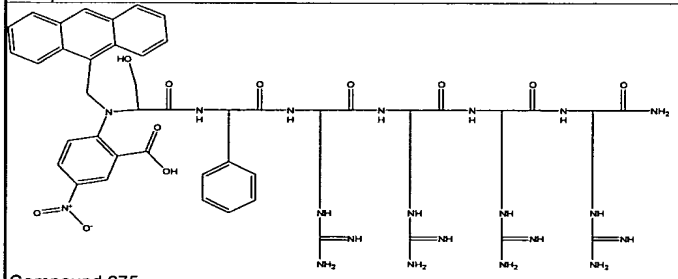
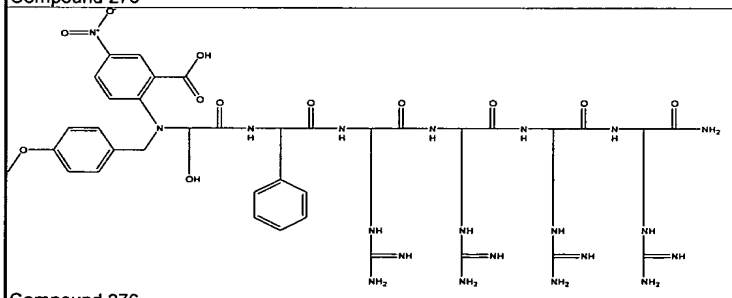
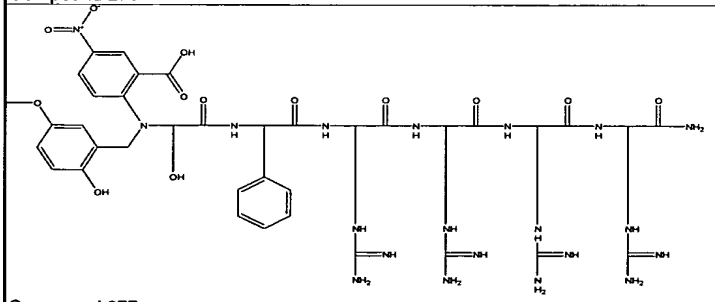
 Compound 248	C58H79N21O13	1278.395	<10%
 Compound 249	C54H75N21O14	1242.3188	<10%
 Compound 250	C60H79N21O13	1302.417	<10%
 Compound 251	C58H77N21O13	1276.3792	<10%
 Compound 252	C58H77N21O13	1276.3792	<10%

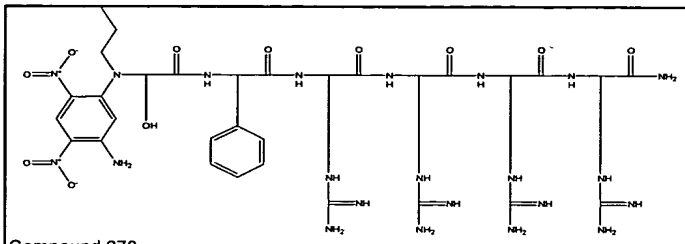
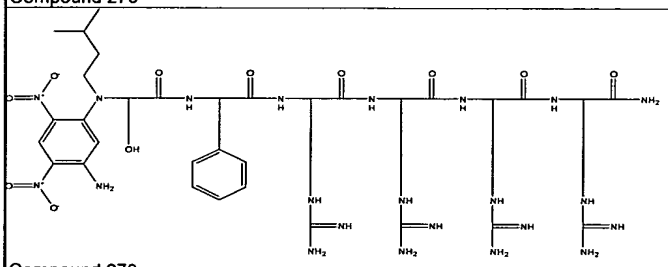
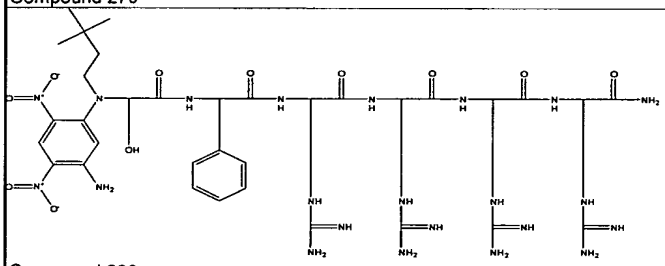
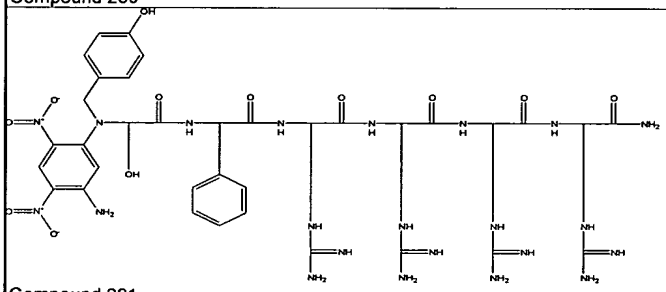
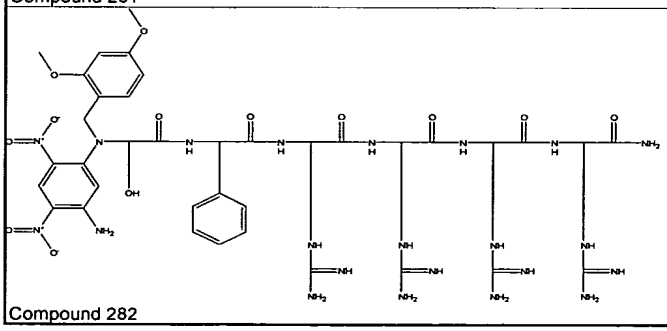
 Compound 253	C51H75N21O14	1206.2858	<10%
 Compound 254	C51H75N21O15	1222.2852	<10%
 Compound 255	C46H74N20O11	1083.218	<10%
 Compound 256	C48H78N20O11	1111.2716	<10%
 Compound 257	C49H80N20O11	1125.2984	<10%

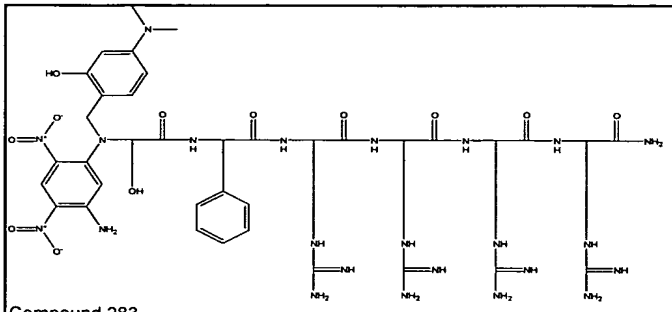
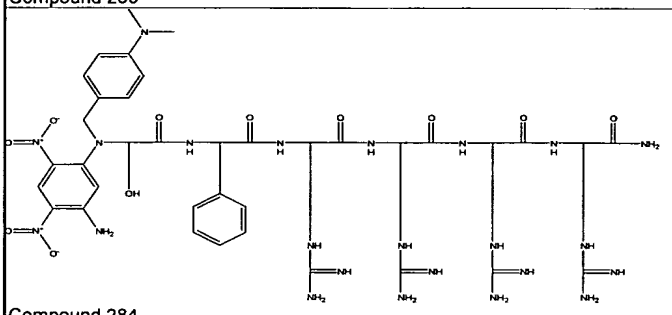
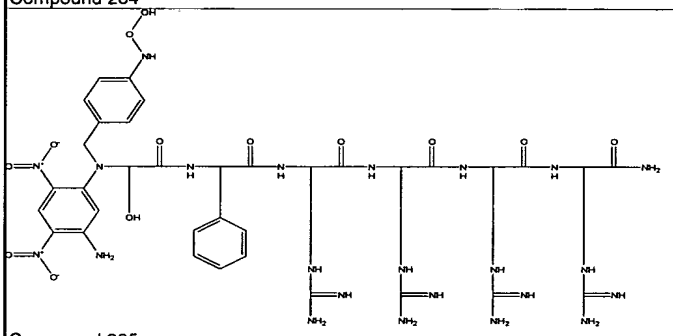
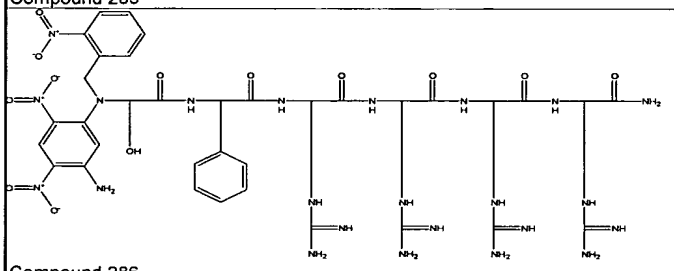
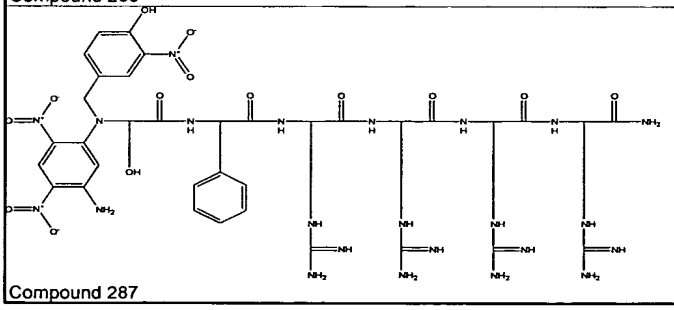
 Compound 258	C50H74N20O12	1147.2614	<10%
 Compound 259	C52H78N20O13	1191.3144	<10%
 Compound 260	C52H79N21O12	1190.3296	<10%
 Compound 261	C52H79N21O11	1174.3302	<10%
 Compound 262	C50H75N21O13	1178.2754	<10%

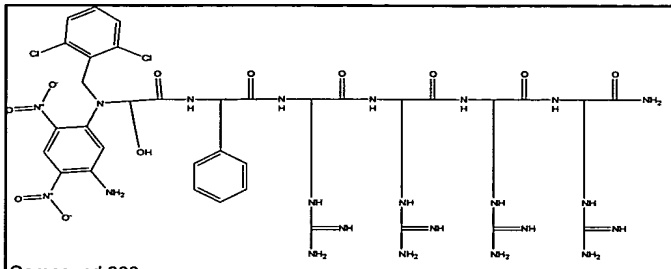
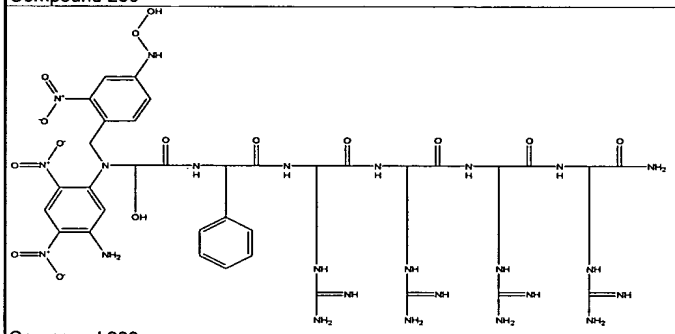
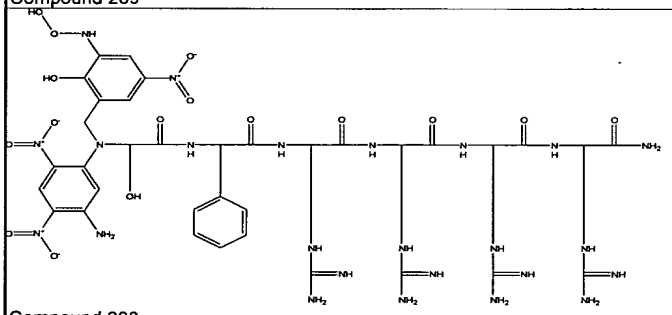
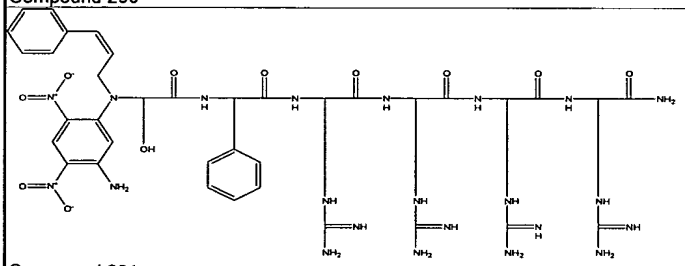
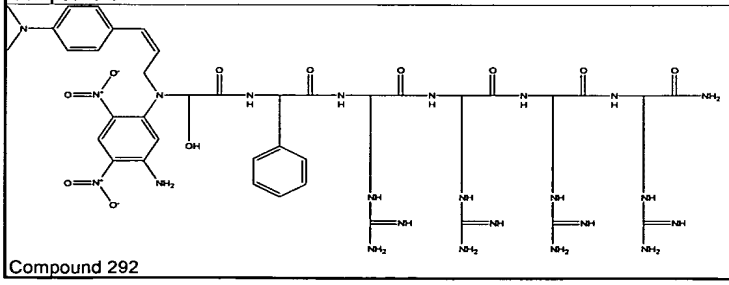
 Compound 263	C50H73N21O13	1176.2596	<10%
 Compound 264	C50H73N21O14	1192.259	<10%
 Compound 265	C50H72Cl2N20O11	1200.1522	<10%
 Compound 266	C50H74N22O15	1223.273	<10%
 Compound 267	C50H74N22O16	1239.2724	<10%

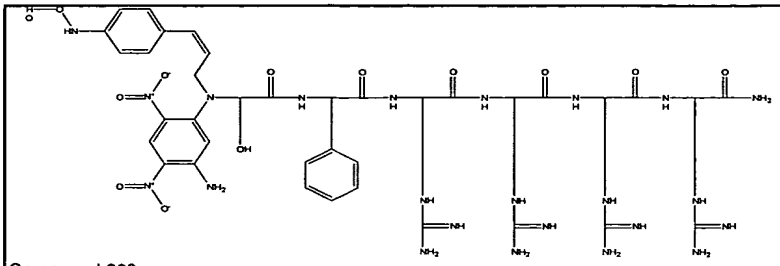
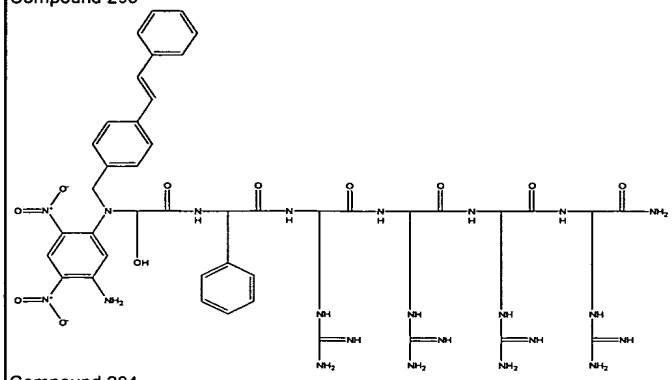
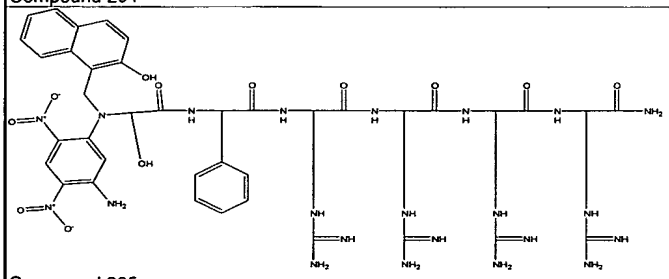
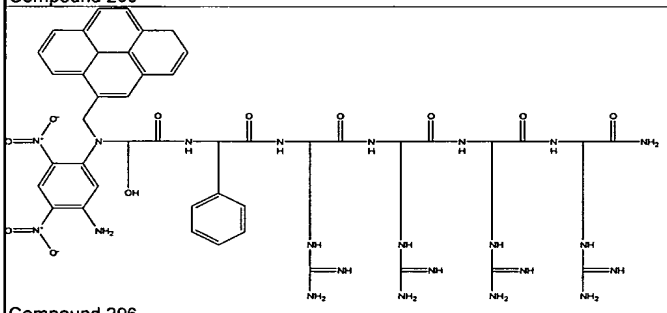
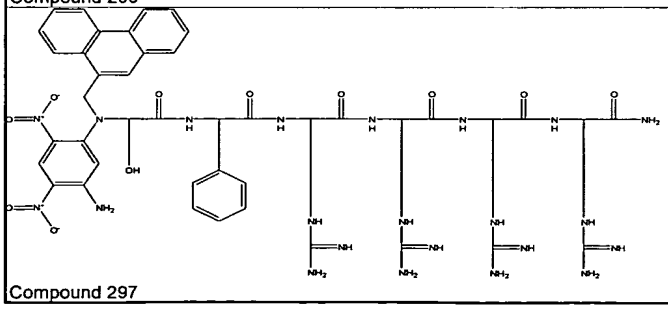
 Compound 268	C52H76N20O11	1157.2998	<10%
 Compound 269	C54H81N21O11	1200.368	<10%
 Compound 270	C52H77N21O13	1204.3132	<10%
 Compound 271	C58H80N20O11	1233.3974	<10%
 Compound 272	C54H76N20O12	1197.3212	<10%

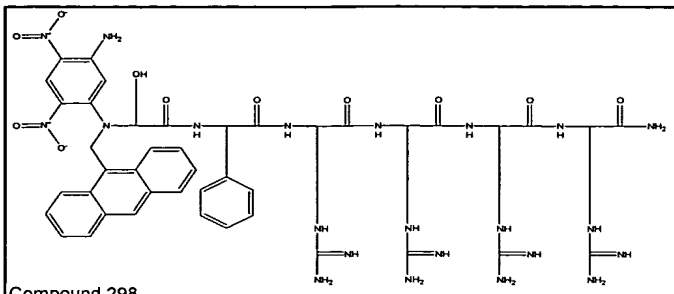
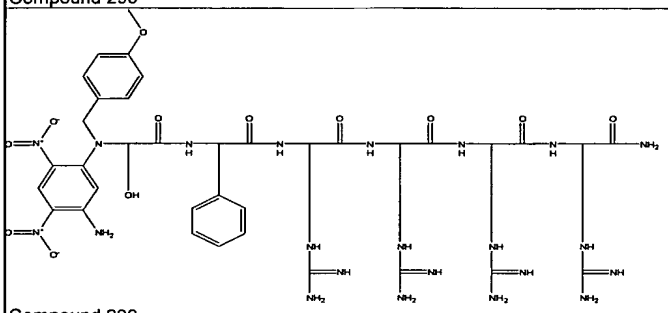
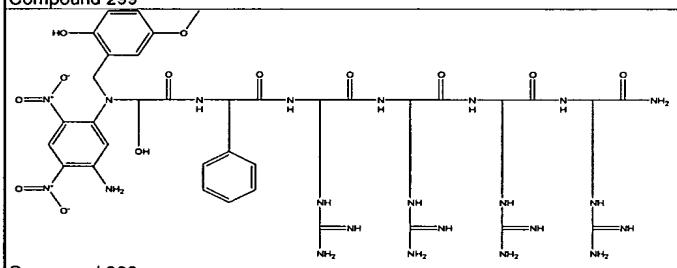
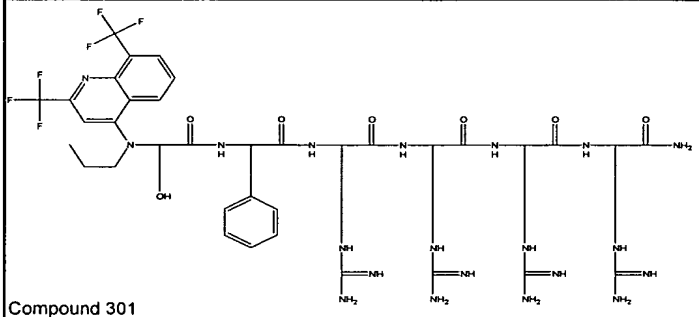
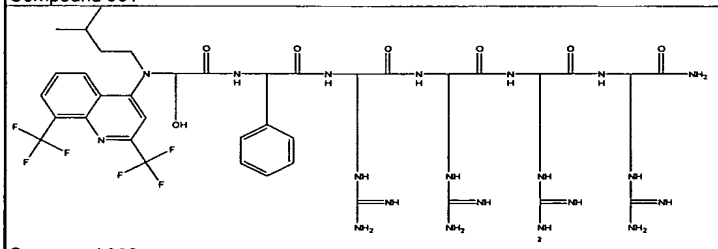
 Compound 273	C60H80N20O11	1257.4194	<10%
 Compound 274	C58H78N20O11	1231.3816	<10%
 Compound 275	C58H78N20O11	1231.3816	<10%
 Compound 276	C51H76N20O12	1161.2882	<10%
 Compound 277	C51H76N20O13	1177.2876	<10%

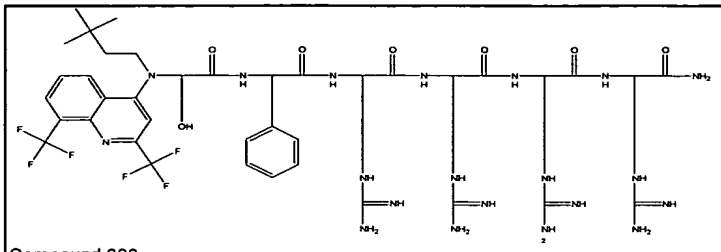
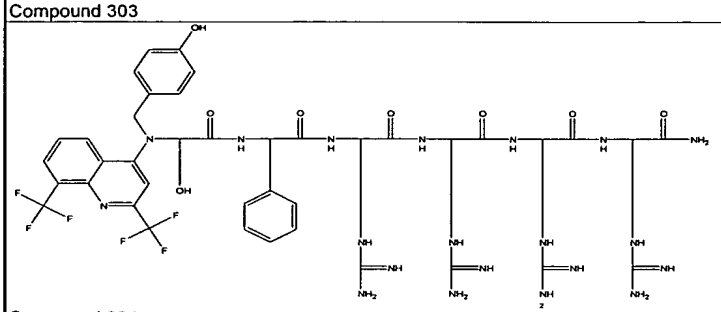
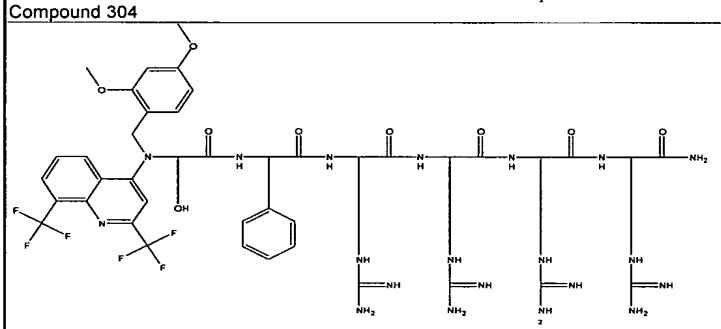
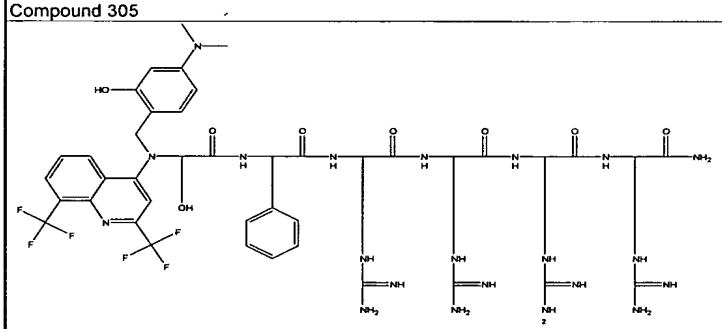
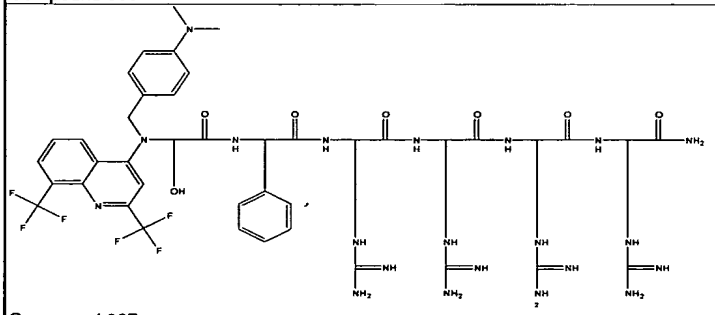
 Compound 278	C45H74N22O11	1099.2204	<10%
 Compound 279	C47H78N22O11	1127.274	<10%
 Compound 280	C48H80N22O11	1141.3008	<10%
 Compound 281	C49H74N22O12	1163.2638	<10%
 Compound 282	C51H78N22O13	1207.3168	<10%

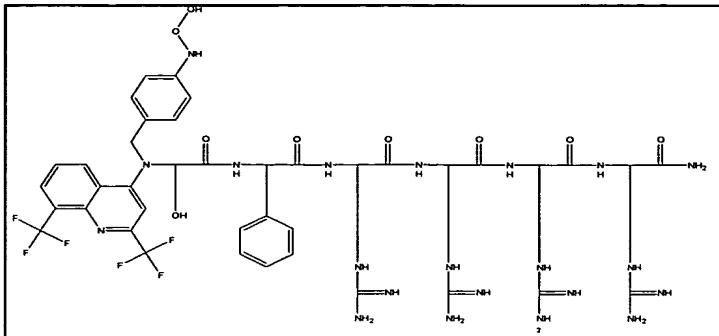
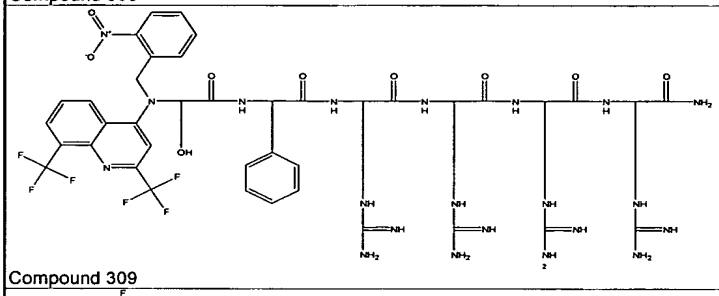
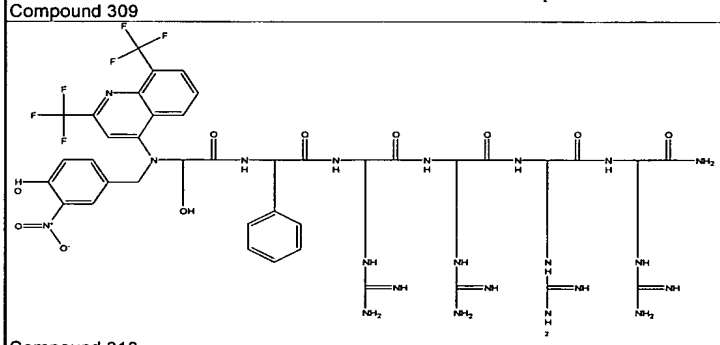
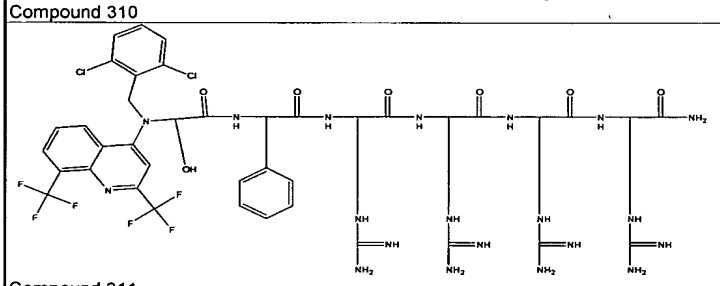
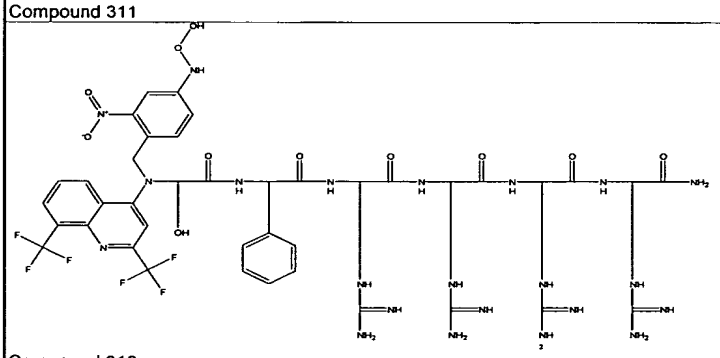
 Compound 283	C51H79N23O12	1206.332	<10%
 Compound 284	C51H79N23O11	1190.3326	<10%
 Compound 285	C49H75N23O13	1194.2778	<10%
 Compound 286	C49H73N23O13	1192.262	<10%
 Compound 287	C49H73N23O14	1208.2614	<10%

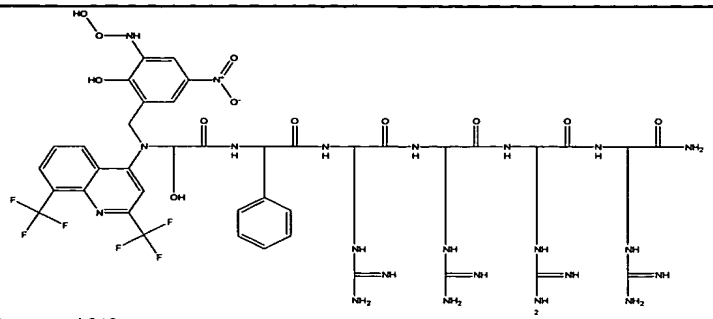
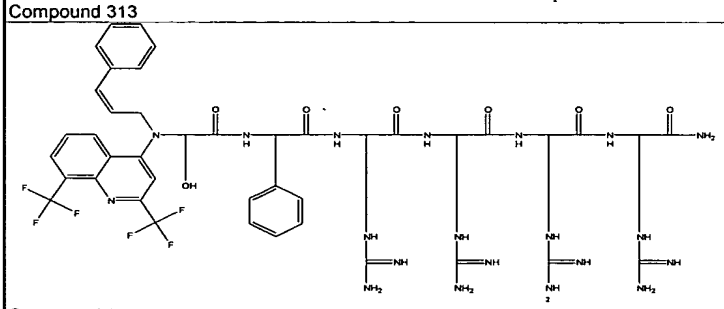
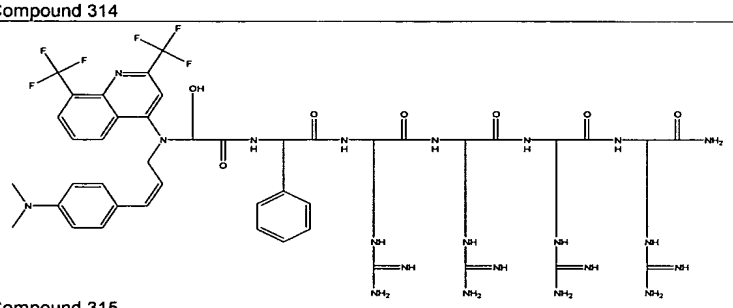
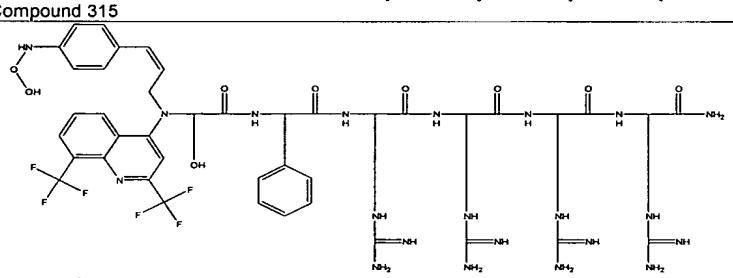
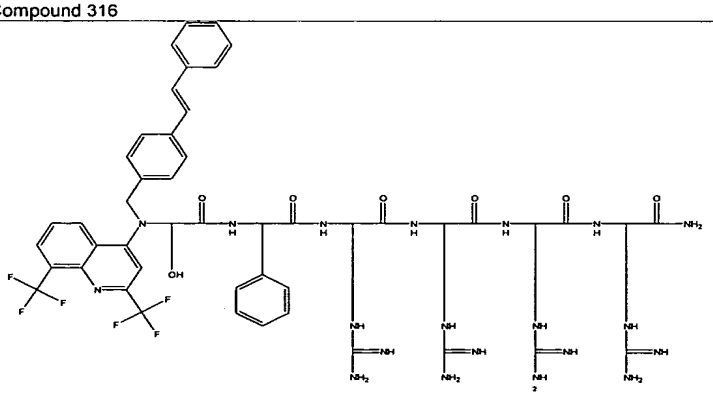
 Compound 288	C49H72Cl2N22O11	1216.1546	<10%
 Compound 289	C49H74N24O15	1239.2754	<10%
 Compound 290	C49H74N24O16	1255.2748	<10%
 Compound 291	C51H76N22O11	1173.3022	<10%
 Compound 292	C53H81N23O11	1216.3704	<10%

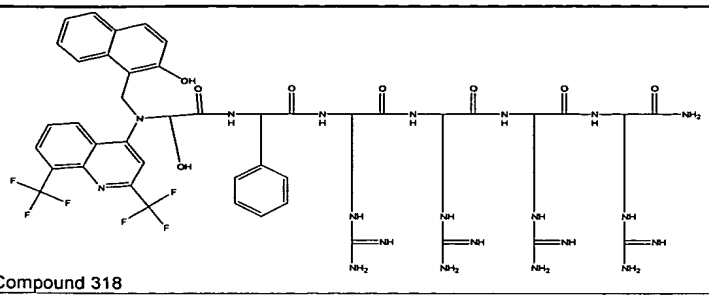
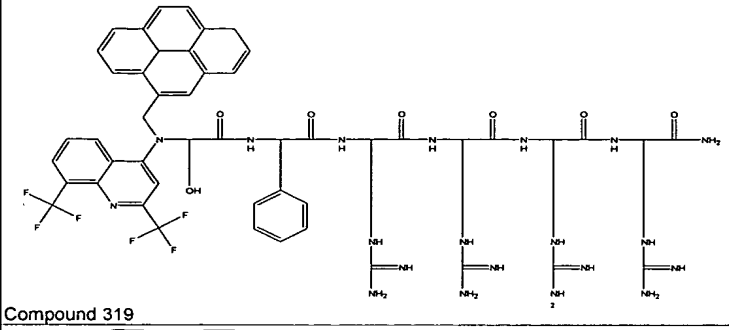
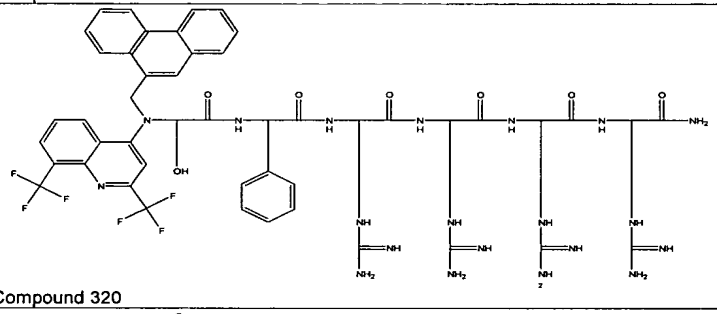
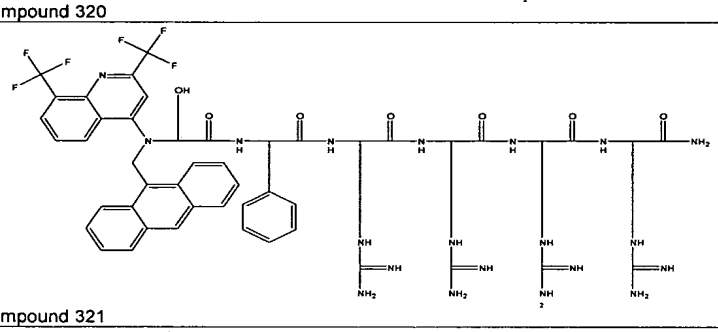
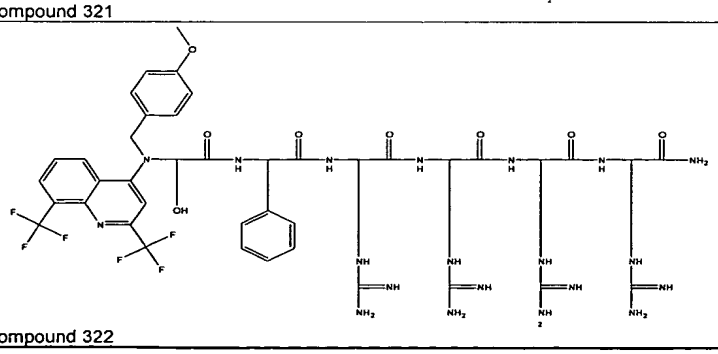
 Compound 293	C51H77N23O13	1220.3156	<10%
 Compound 294	C57H80N22O11	1249.3998	<10%
 Compound 295	C53H76N22O12	1213.3236	<10%
 Compound 296	C59H80N22O11	1273.4218	<10%
 Compound 297	C57H78N22O11	1247.384	<10%

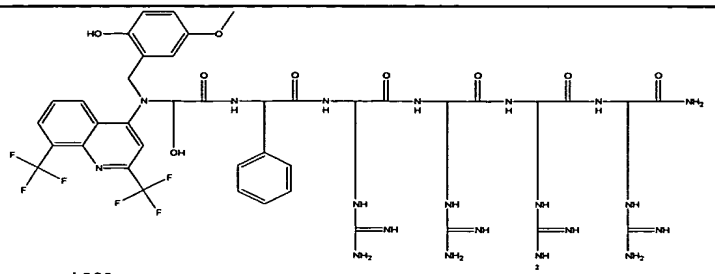
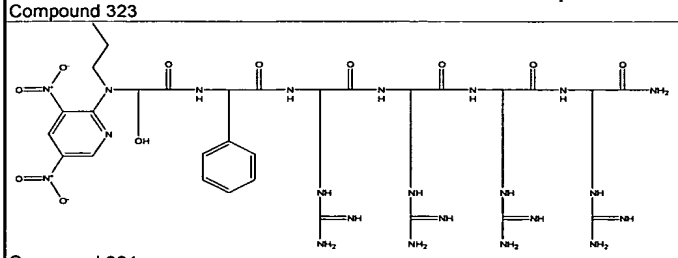
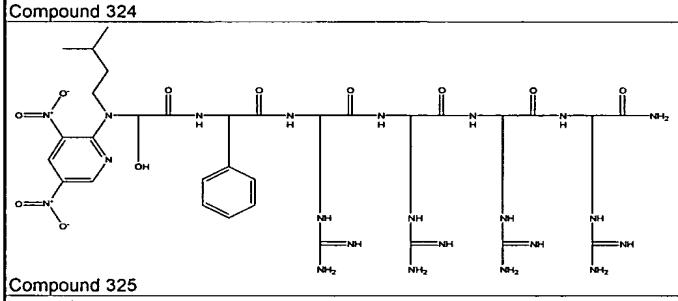
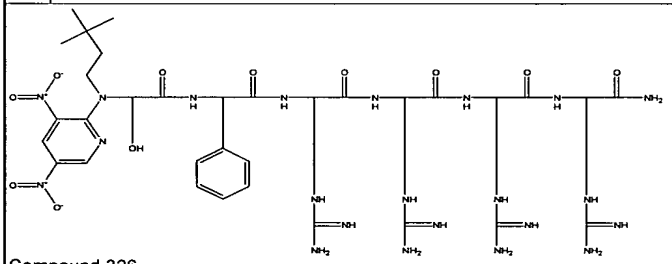
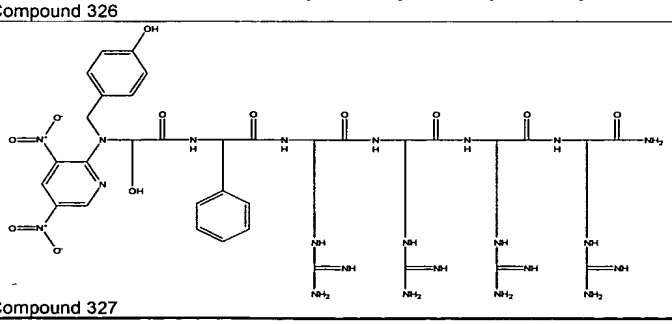
 Compound 298	C57H78N22O11	1247.384	<10%
 Compound 299	C50H76N22O12	1177.2906	<10%
 Compound 300	C50H76N22O13	1193.29	<10%
 Compound 301	C50H74F6N20O7	1181.2548	<10%
 Compound 302	C52H78F6N20O7	1209.3084	<10%

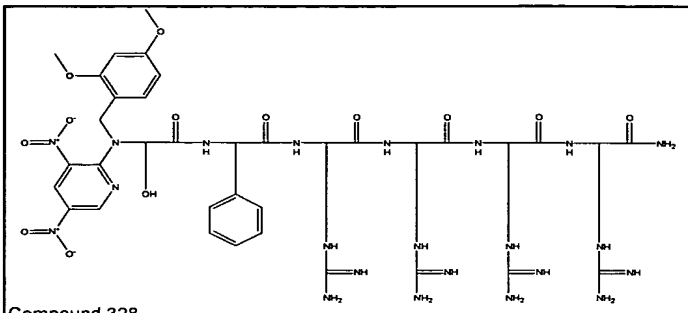
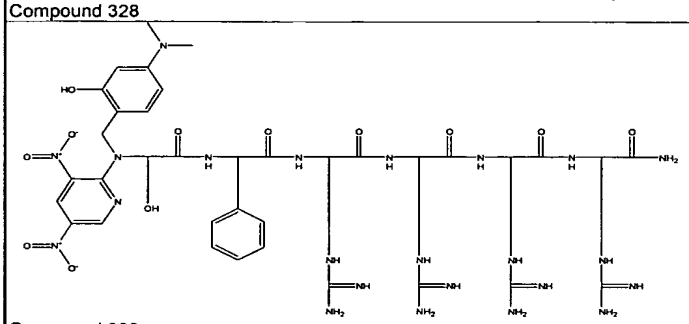
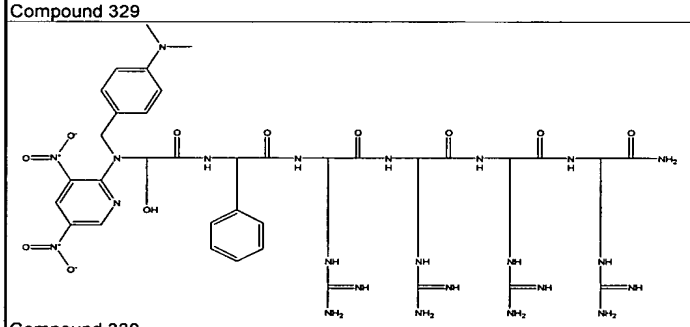
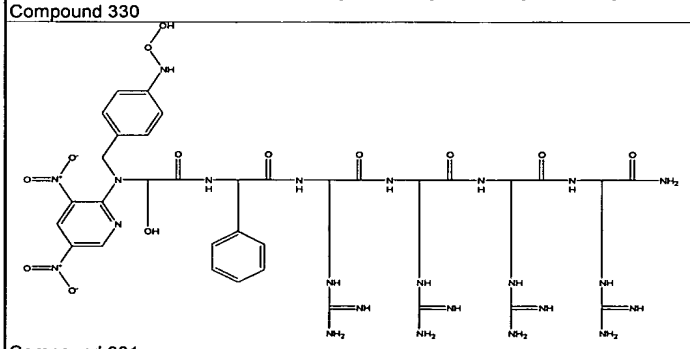
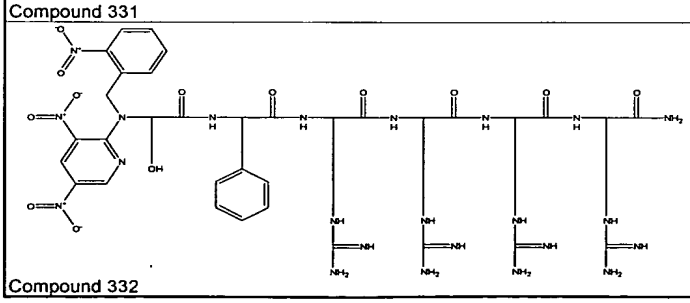
 Compound 303	C53H80F6N20O7	1223.3352	<10%
 Compound 304	C54H74F6N20O8	1245.2982	<10%
 Compound 305	C56H78F6N20O9	1289.3512	<10%
 Compound 306	C56H79F6N21O8	1288.3664	<10%
 Compound 307	C56H79F6N21O7	1272.367	<10%

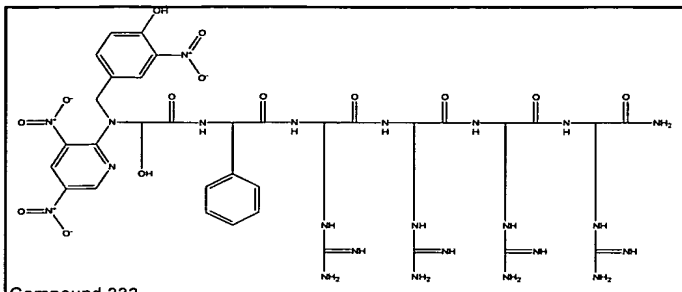
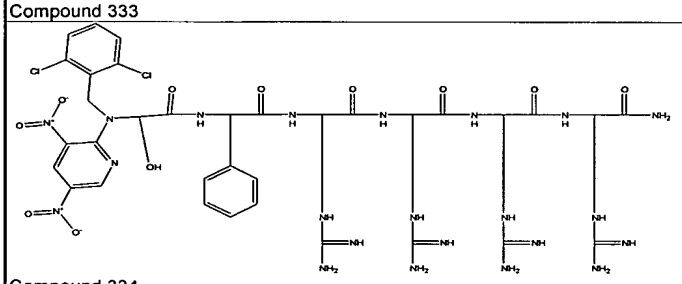
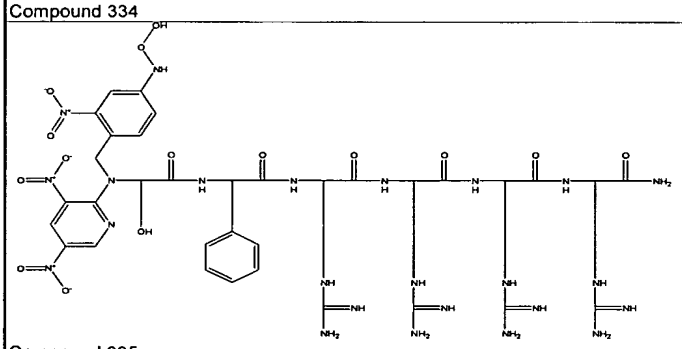
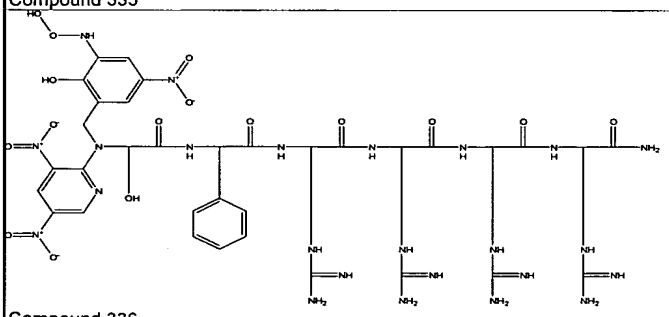
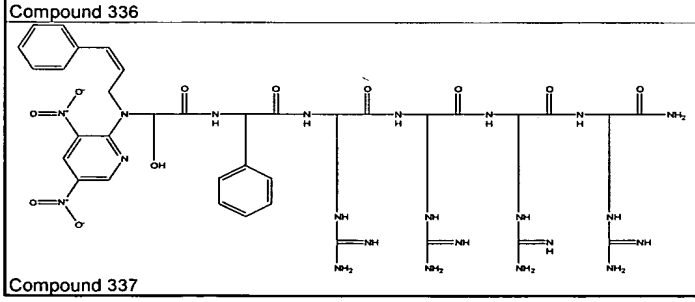
 Compound 308	C54H75F6N21O9	1276.3122	<10%
 Compound 309	C54H73F6N21O9	1274.2964	<10%
 Compound 310	C54H73F6N21O10	1290.2958	<10%
 Compound 311	C54H72Cl2F6N20O7	1298.189	<10%
 Compound 312	C54H74F6N22O11	1321.3098	<10%

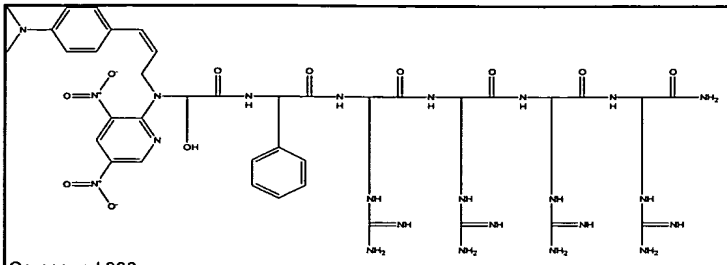
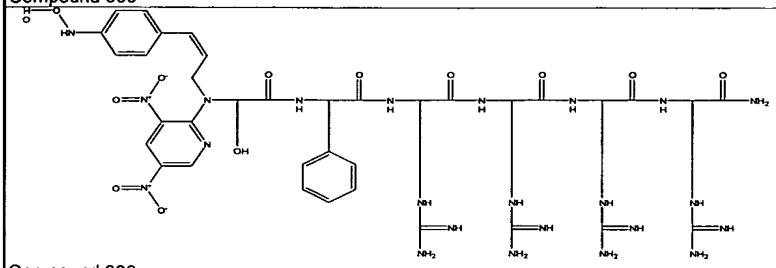
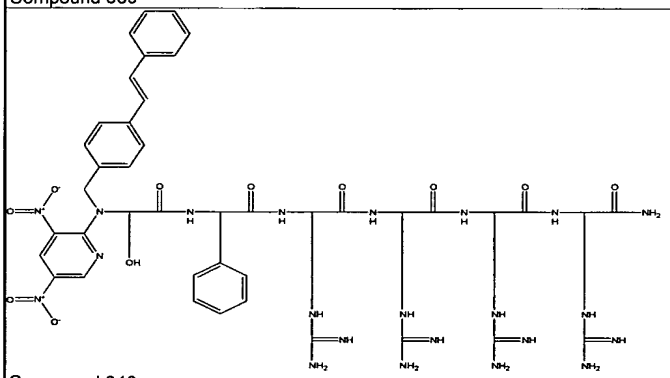
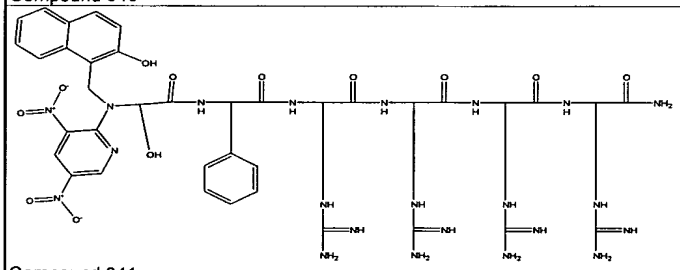
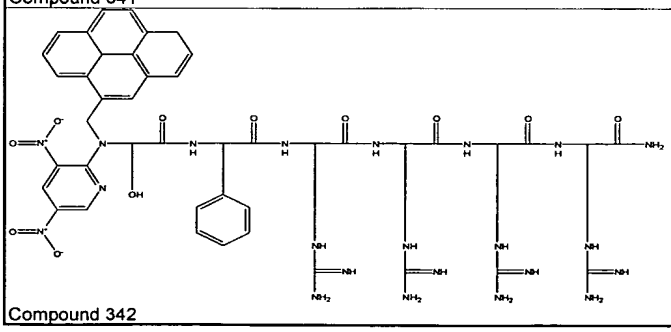
 Compound 313	C54H74F6N22O12	1337.3092	<10%
 Compound 314	C56H76F6N20O7	1255.3366	<10%
 Compound 315	C58H81F6N21O7	1298.4048	<10%
 Compound 316	C56H77F6N21O9	1302.35	<10%
 Compound 317	C62H80F6N20O7	1331.4342	<10%

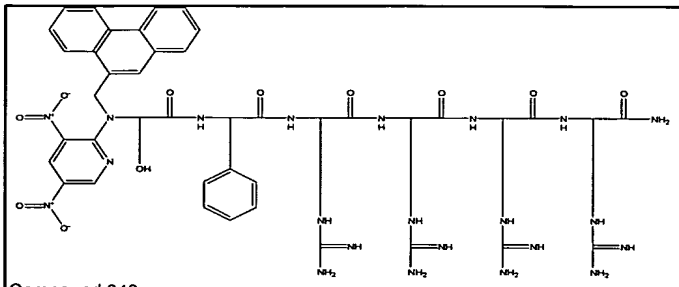
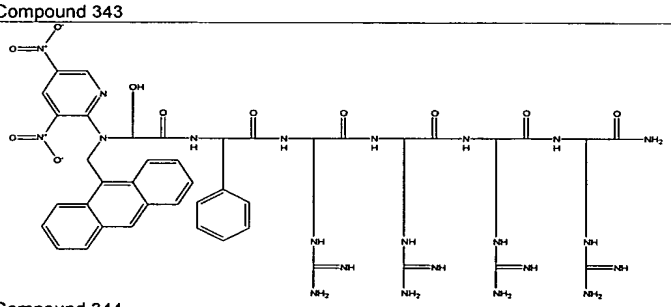
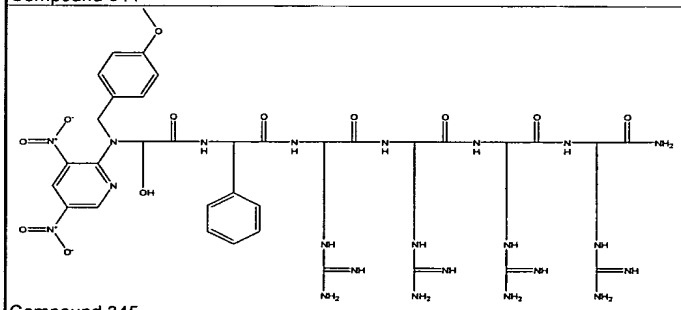
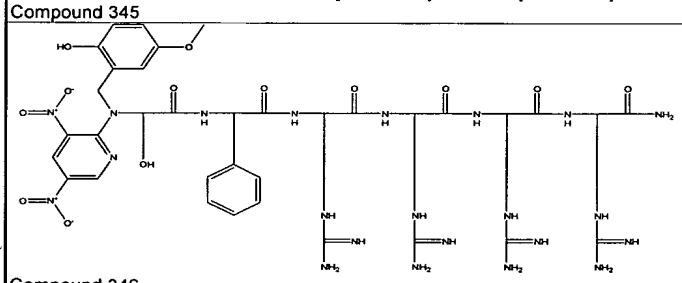
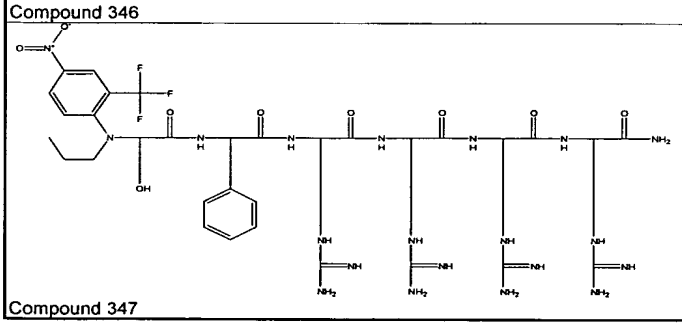
 Compound 318	C58H76F6N20O8	1295.358	<10%
 Compound 319	C64H80F6N20O7	1355.4562	<10%
 Compound 320	C62H78F6N20O7	1329.4184	<10%
 Compound 321	C62H78F6N20O7	1329.4184	<10%
 Compound 322	C55H76F6N20O8	1259.325	<10%

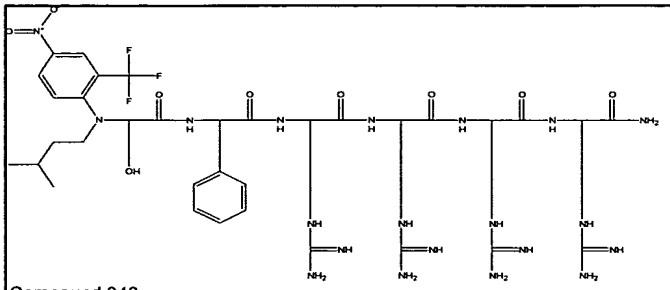
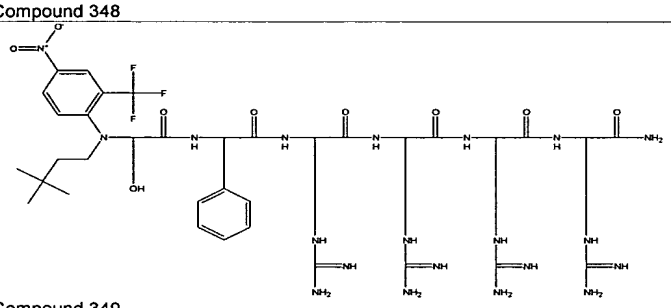
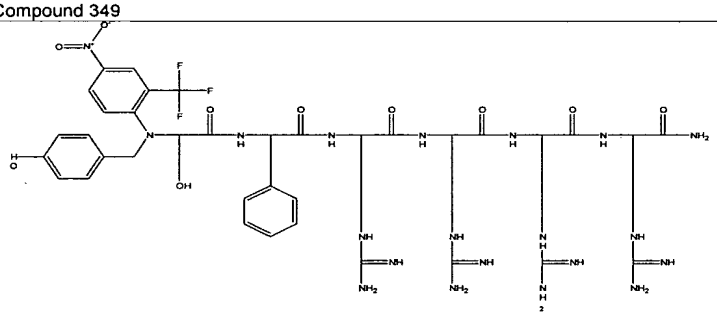
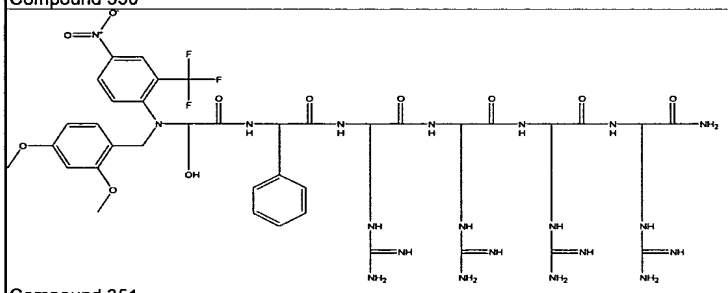
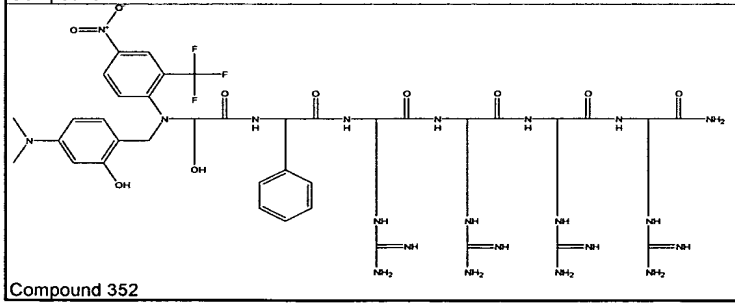
 Compound 323	C55H76F6N20O9	1275.3244	<10%
 Compound 324	C44H72N22O11	1085.1936	<10%
 Compound 325	C46H76N22O11	1113.2472	<10%
 Compound 326	C47H78N22O11	1127.274	<10%
 Compound 327	C48H72N22O12	1149.237	<10%

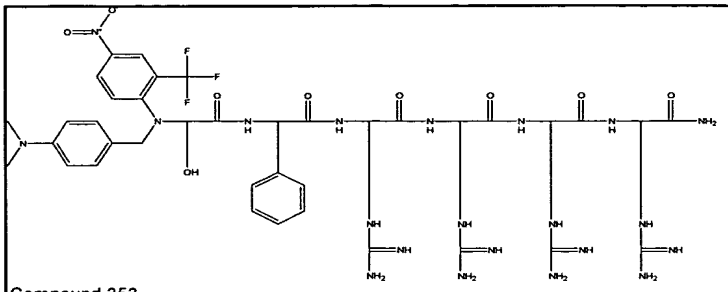
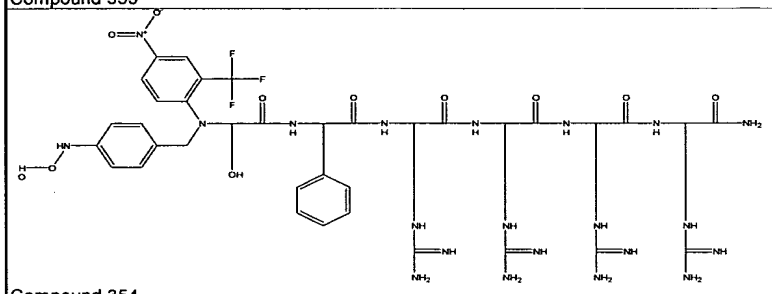
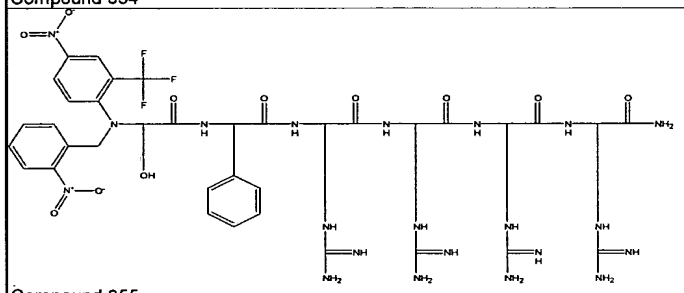
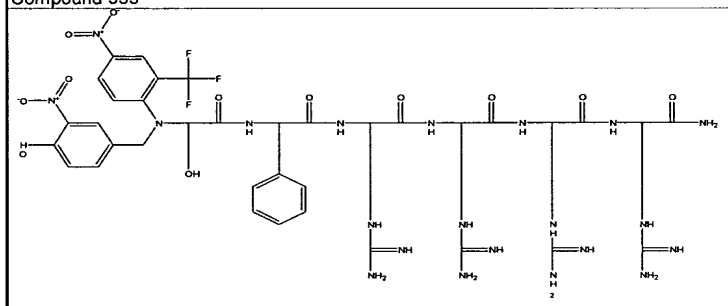
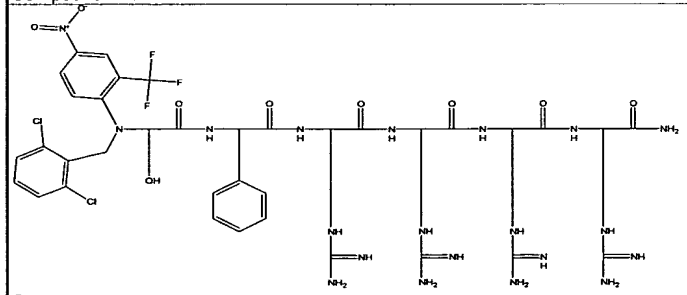
 Compound 328	C50H76N22O13	1193.29	<10%
 Compound 329	C50H77N23O12	1192.3052	<10%
 Compound 330	C50H77N23O11	1176.3058	<10%
 Compound 331	C48H73N23O13	1180.251	<10%
 Compound 332	C48H71N23O13	1178.2352	<10%

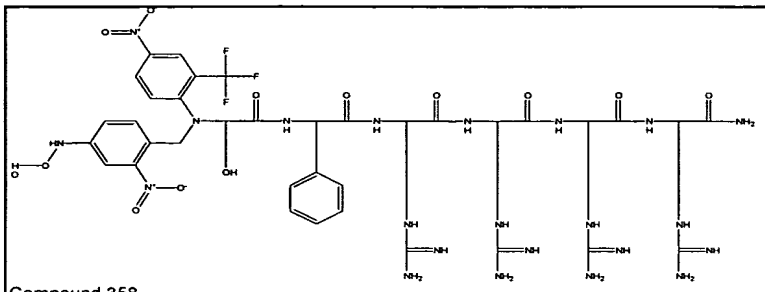
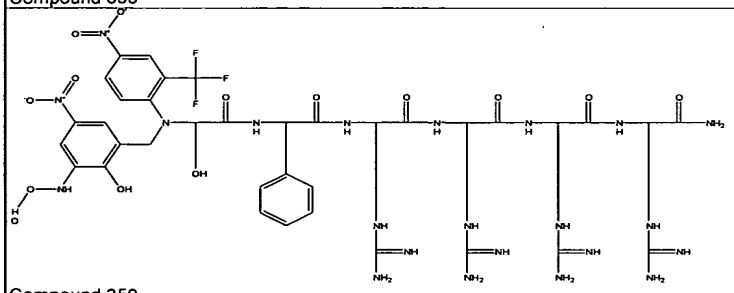
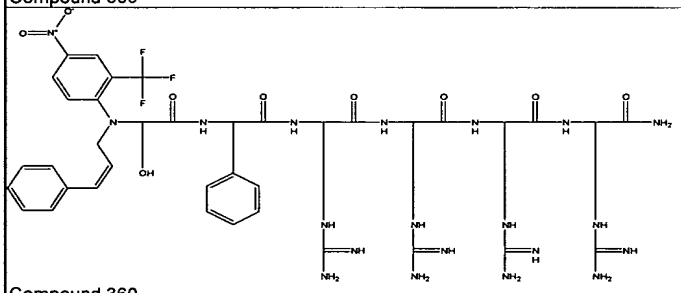
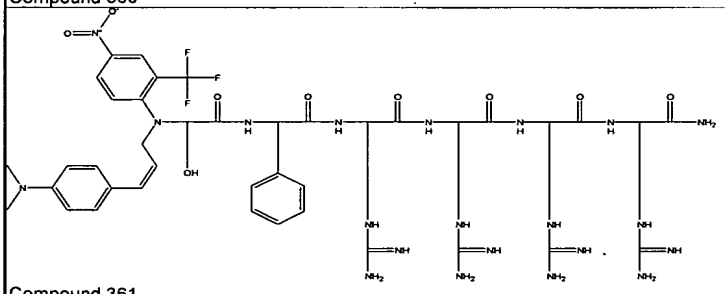
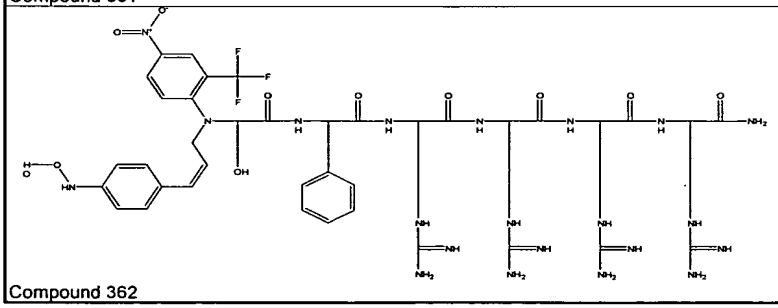
 Compound 333	C48H71N23O14	1194.2346	<10%
 Compound 334	C48H70Cl2N22O11	1202.1278	<10%
 Compound 335	C48H72N24O15	1225.2486	<10%
 Compound 336	C48H72N24O16	1241.248	<10%
 Compound 337	C50H74N22O11	1159.2754	<10%

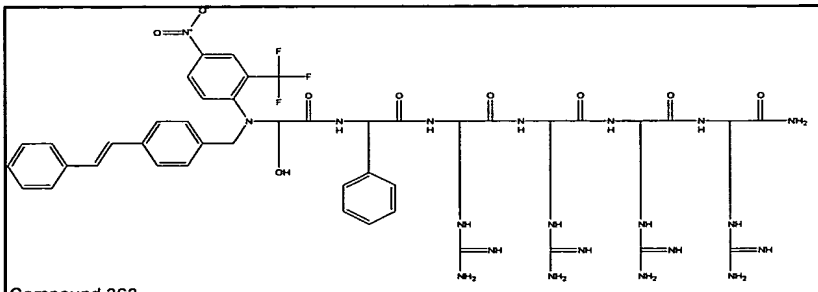
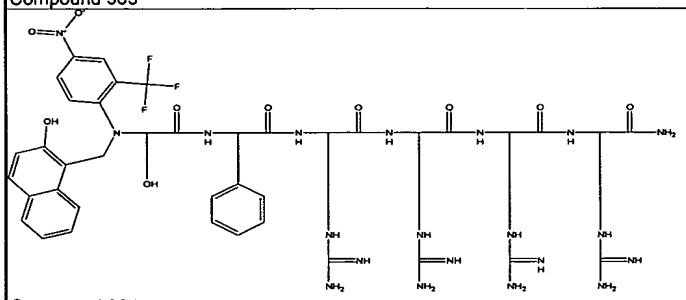
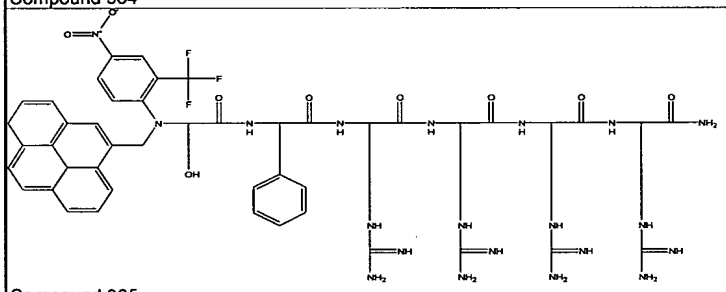
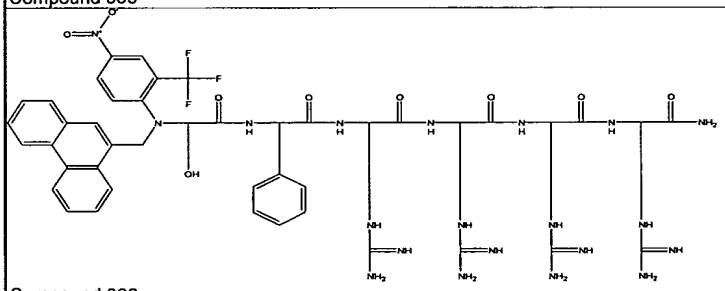
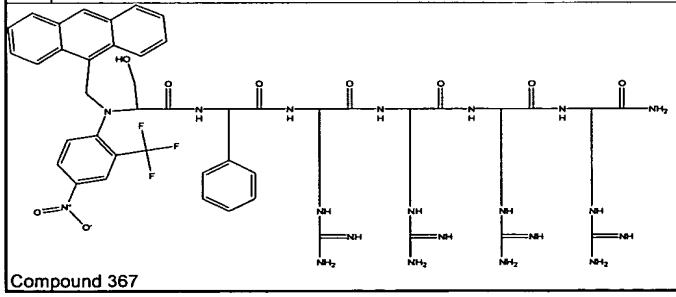
 Compound 338	C52H79N23O11	1202.3436	<10%
 Compound 339	C50H75N23O13	1206.2888	<10%
 Compound 340	C56H78N22O11	1235.373	<10%
 Compound 341	C52H74N22O12	1199.2968	<10%
 Compound 342	C58H78N22O11	1259.395	<10%

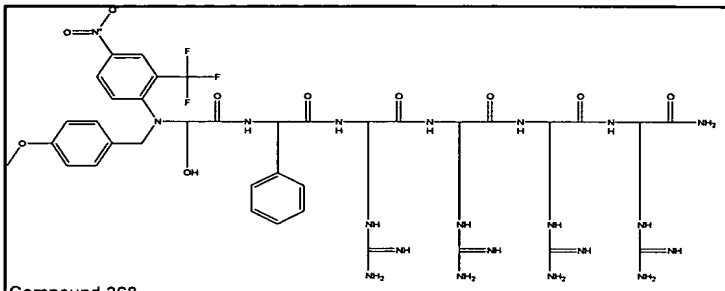
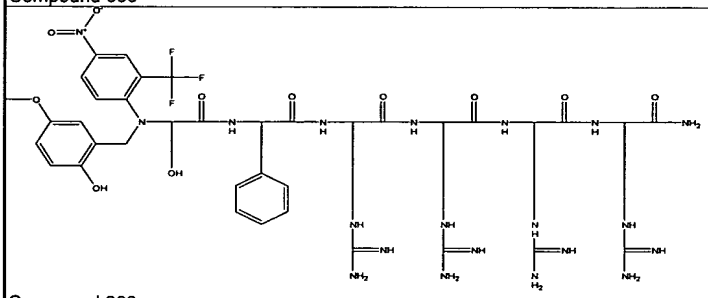
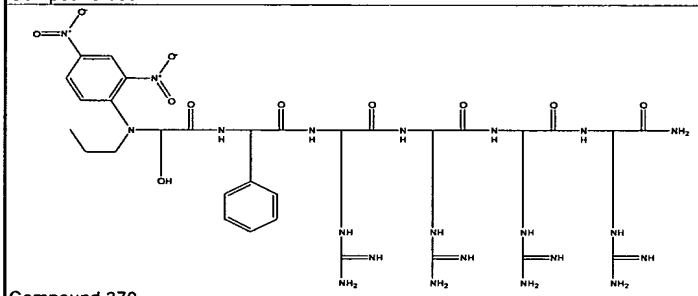
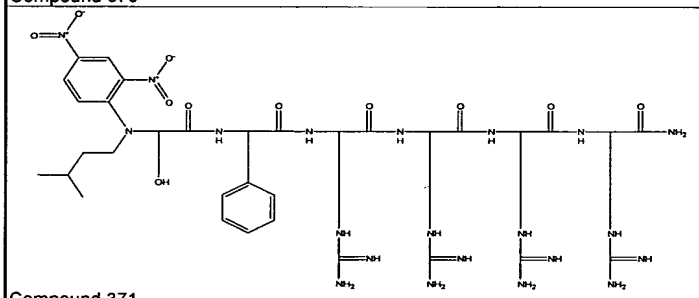
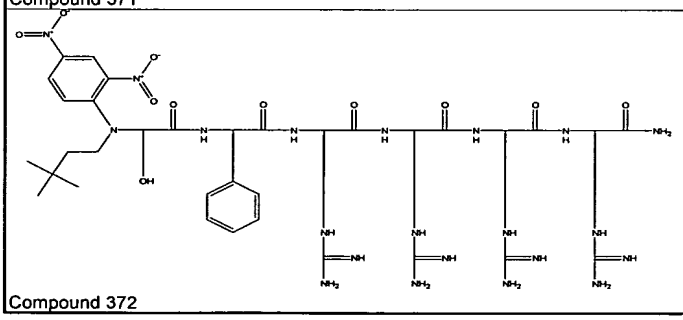
 Compound 343	C56H76N22O11	1233.3572	<10%
 Compound 344	C56H76N22O11	1233.3572	<10%
 Compound 345	C49H74N22O12	1163.2638	<10%
 Compound 346	C49H74N22O13	1179.2632	<10%
 Compound 347	C46H73F3N20O9	1107.2065	<10%

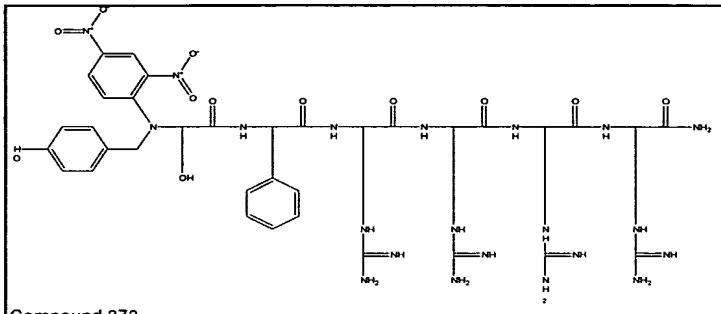
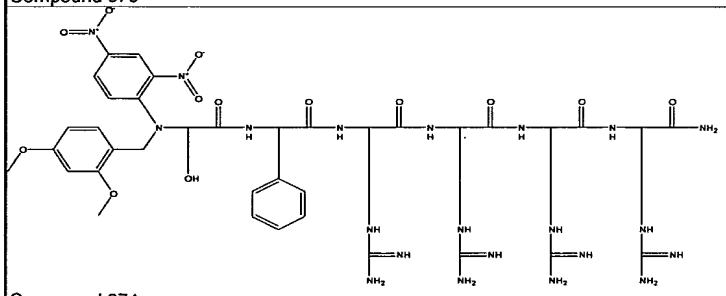
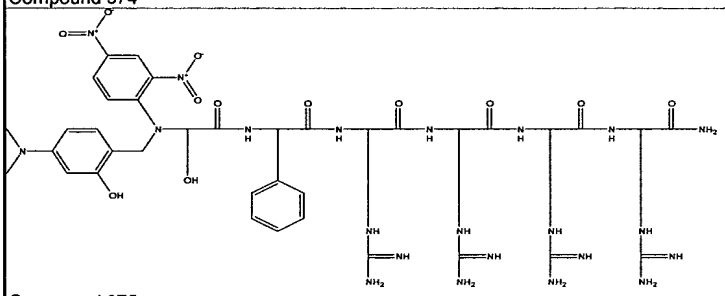
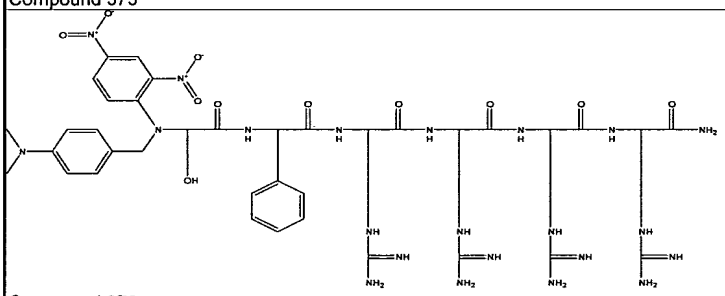
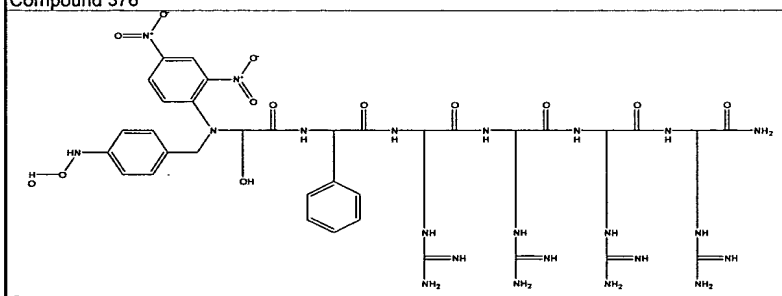
 Compound 348	C48H77F3N20O9	1135.2601	<10%
 Compound 349	C49H79F3N20O9	1149.2869	<10%
 Compound 350	C50H73F3N20O10	1171.2499	<10%
 Compound 351	C52H77F3N20O11	1215.3029	<10%
 Compound 352	C52H78F3N21O10	1214.3181	<10%

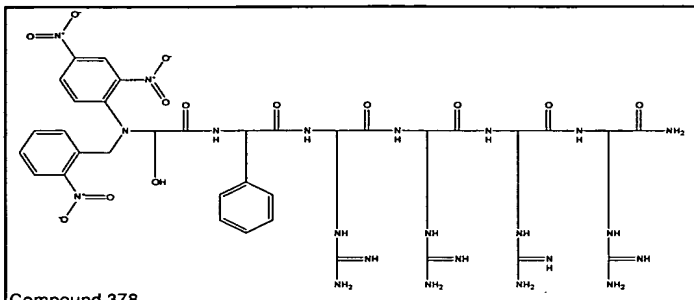
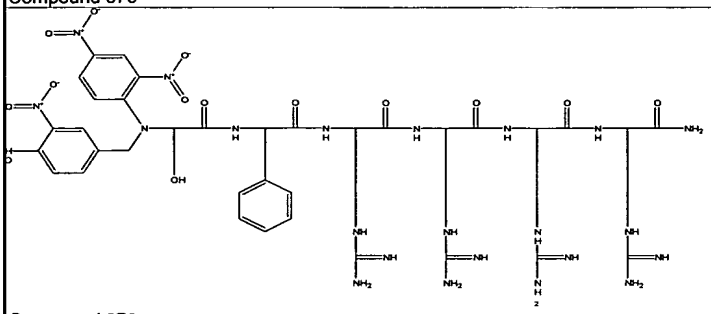
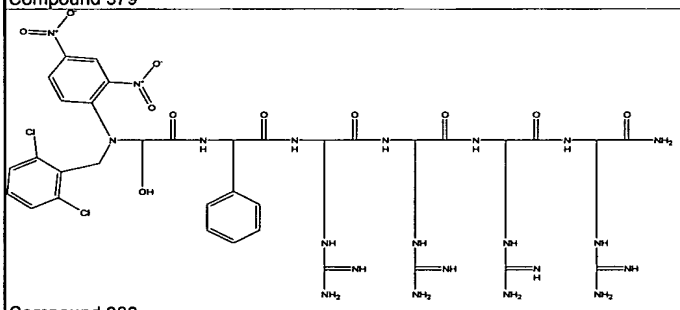
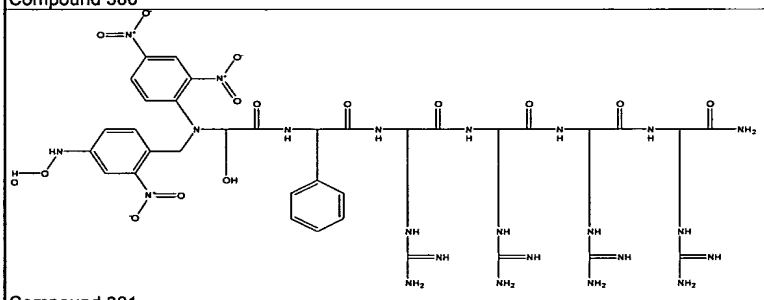
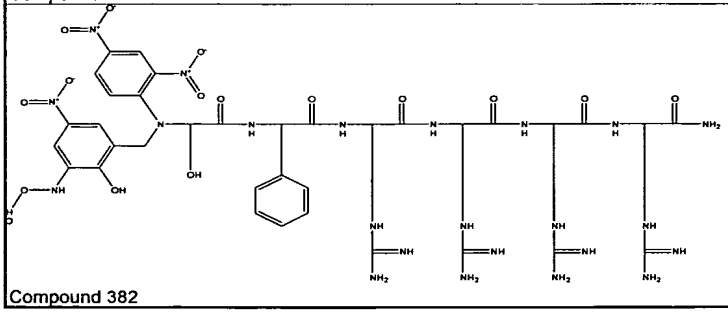
 Compound 353	C52H78F3N21O9	1198.3187	<10%
 Compound 354	C50H74F3N21O11	1202.2639	<10%
 Compound 355	C50H72F3N21O11	1200.2481	<10%
 Compound 356	C50H72F3N21O12	1216.2475	<10%
 Compound 357	C50H71Cl2F3N20O9	1224.1407	<10%

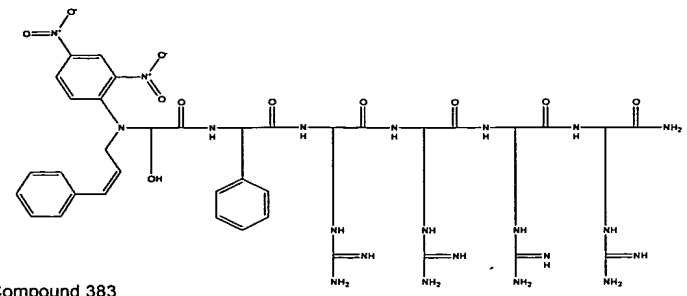
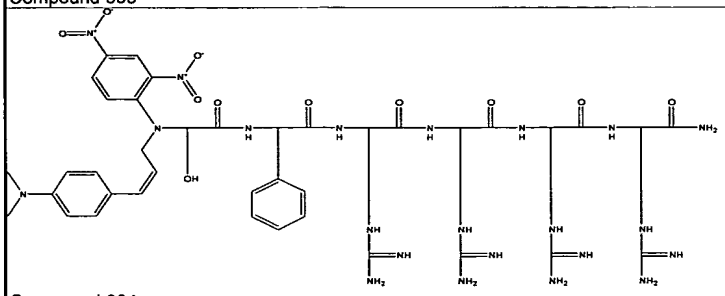
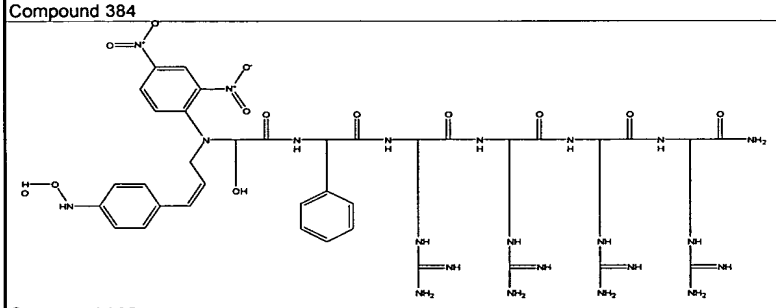
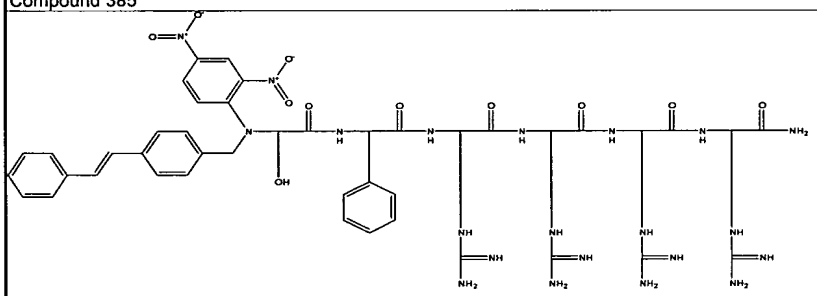
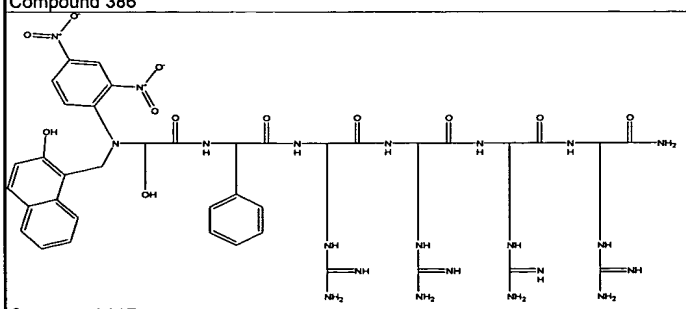
 Compound 358	C50H73F3N22O13	1247.2615	<10%
 Compound 359	C50H73F3N22O14	1263.2609	<10%
 Compound 360	C52H75F3N20O9	1181.2883	<10%
 Compound 361	C54H80F3N21O9	1224.3565	<10%
 Compound 362	C52H76F3N21O11	1228.3017	<10%

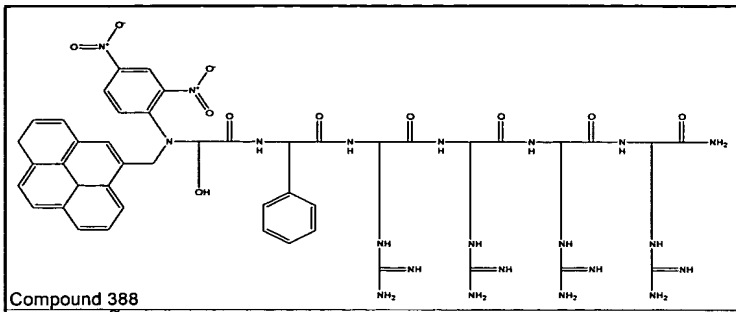
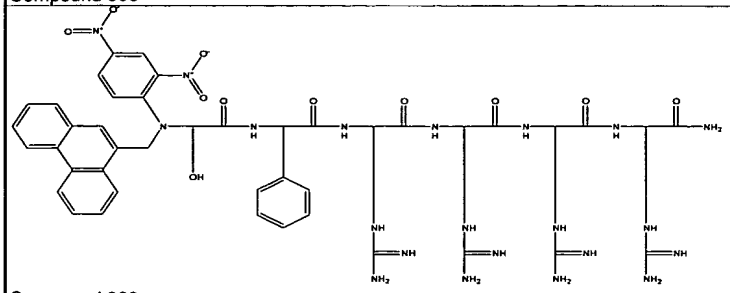
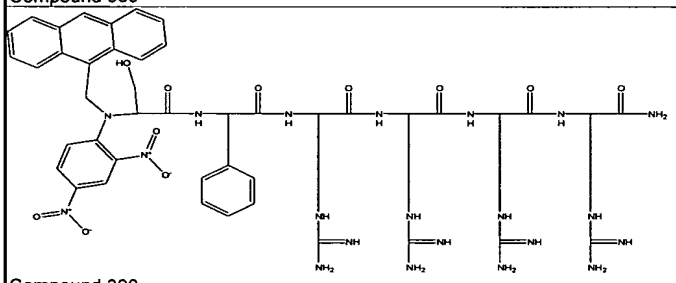
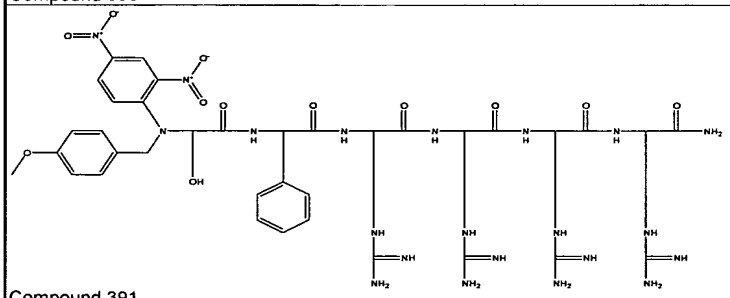
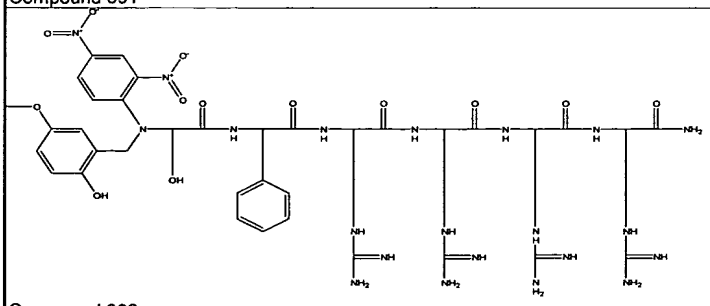
 Compound 363	C58H79F3N20O9	1257.3859	<10%
 Compound 364	C54H75F3N20O10	1221.3097	<10%
 Compound 365	C60H79F3N20O9	1281.4079	<10%
 Compound 366	C58H77F3N20O9	1255.3701	<10%
 Compound 367	C58H77F3N20O9	1255.3701	<10%

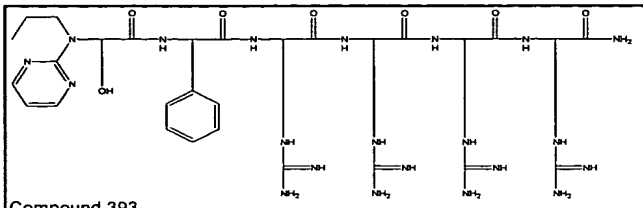
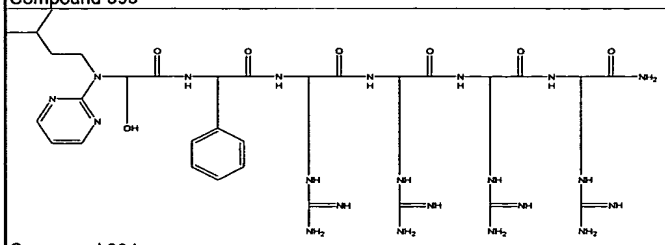
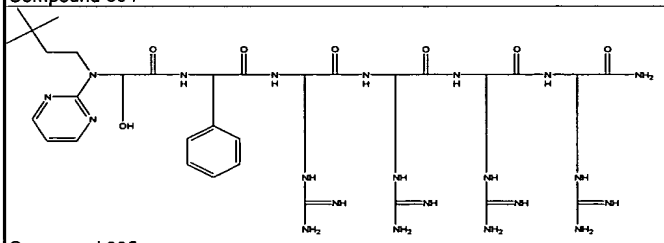
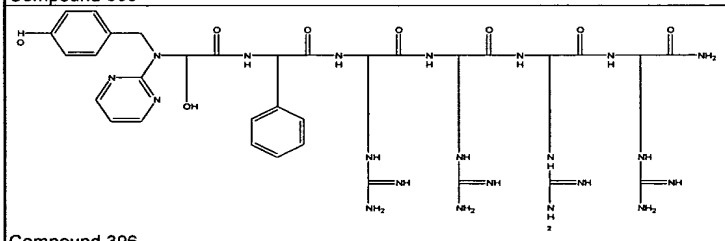
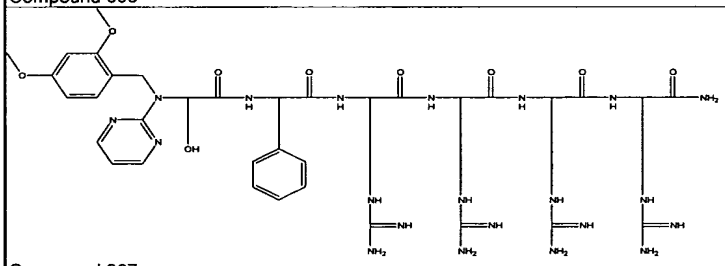
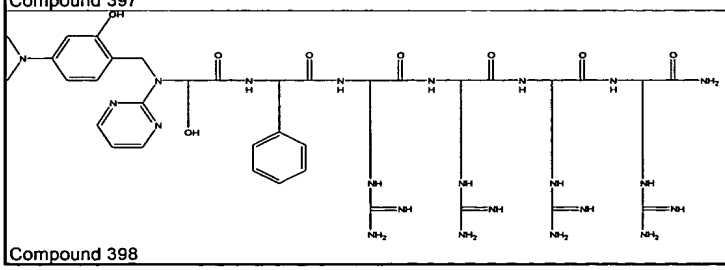
 Compound 368	C51H75F3N20O10	1185.2767	<10%
 Compound 369	C51H75F3N20O11	1201.2761	<10%
 Compound 370	C45H73N21O11	1084.2058	<10%
 Compound 371	C47H77N21O11	1112.2594	<10%
 Compound 372	C48H79N21O11	1126.2862	<10%

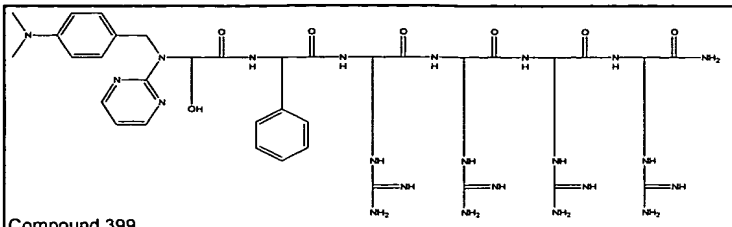
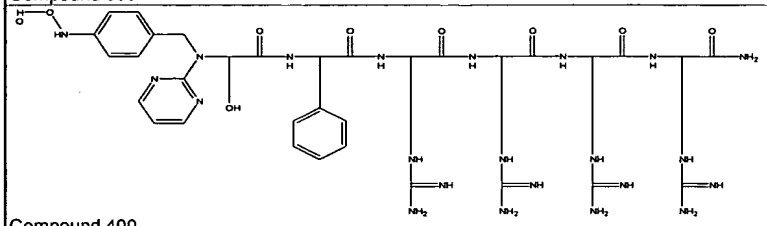
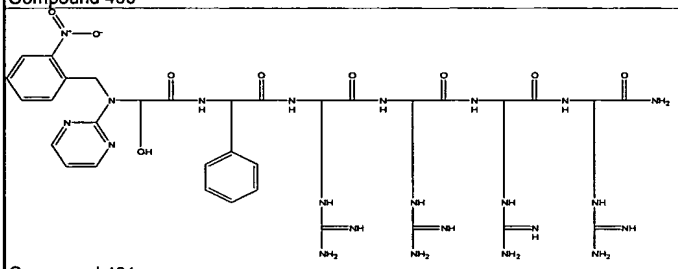
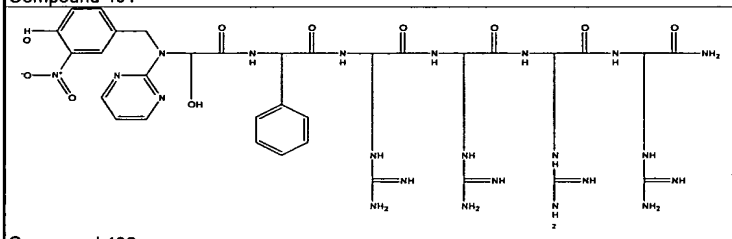
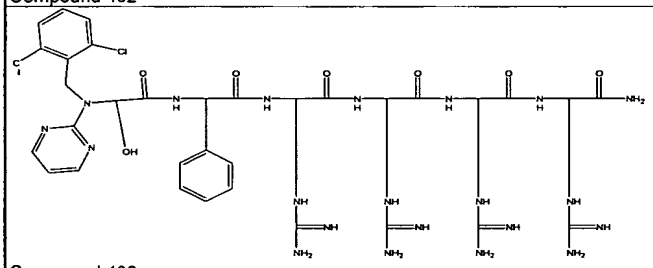
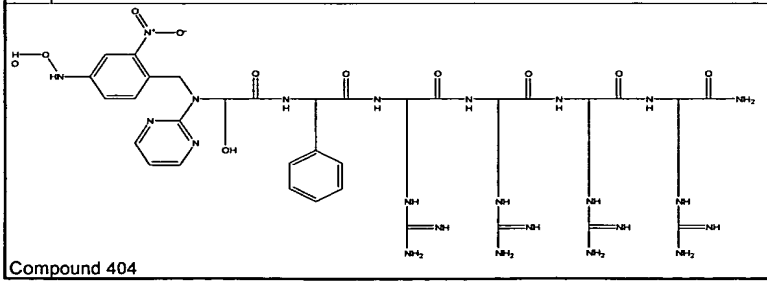
 Compound 373	C49H73N21O12	1148.2492	<10%
 Compound 374	C51H77N21O13	1192.3022	<10%
 Compound 375	C51H78N22O12	1191.3174	<10%
 Compound 376	C51H78N22O11	1175.318	<10%
 Compound 377	C49H74N22O13	1179.2632	<10%

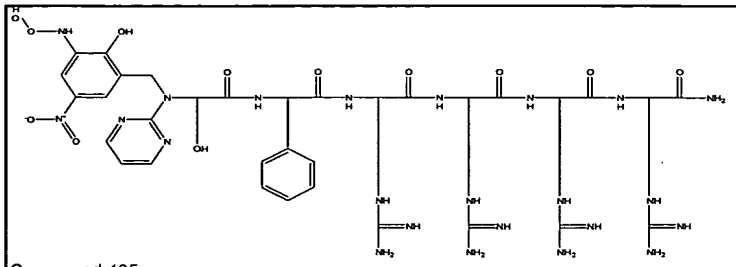
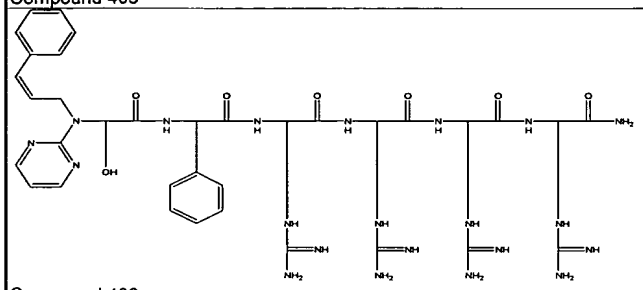
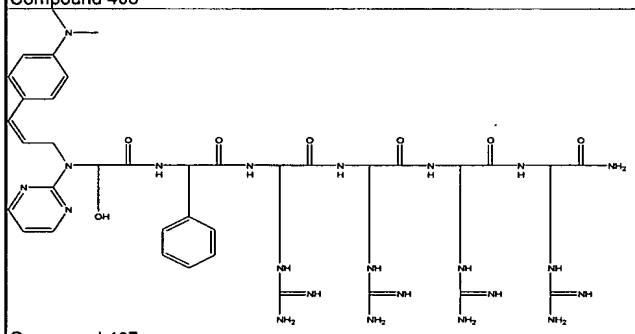
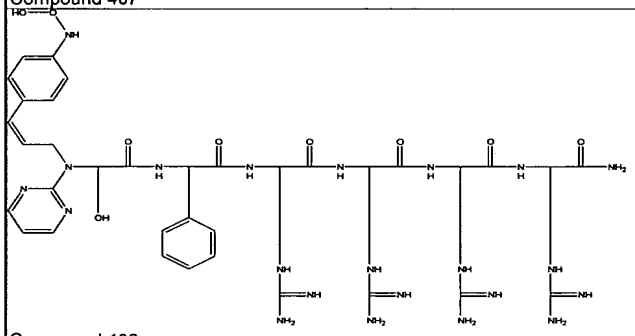
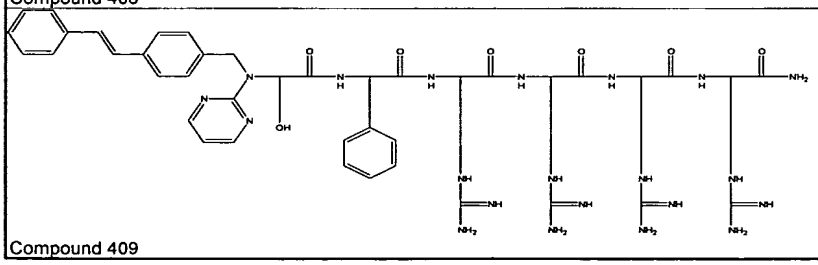
 Compound 378	C49H72N22O13	1177.2474	<10%
 Compound 379	C49H72N22O14	1193.2468	<10%
 Compound 380	C49H71Cl2N21O11	1201.14	<10%
 Compound 381	C49H73N23O15	1224.2608	<10%
 Compound 382	C49H73N23O16	1240.2602	<10%

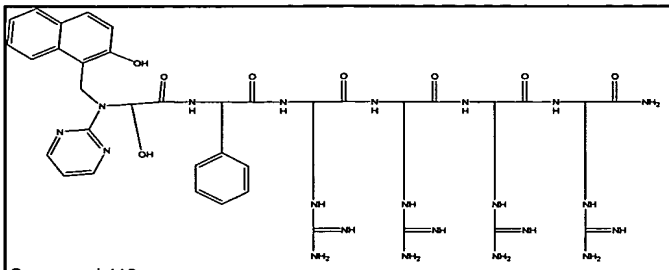
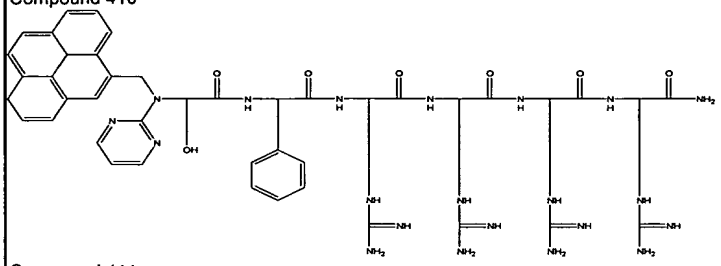
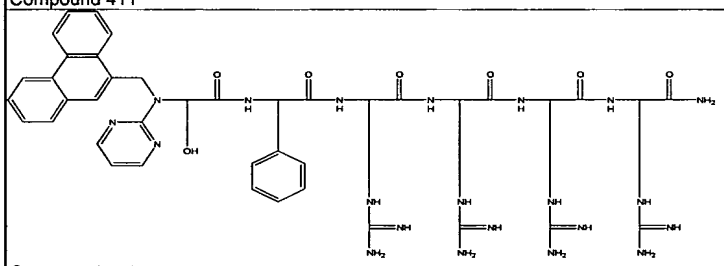
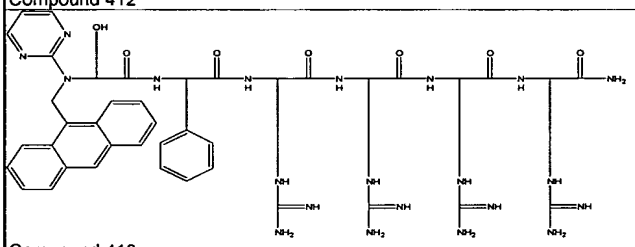
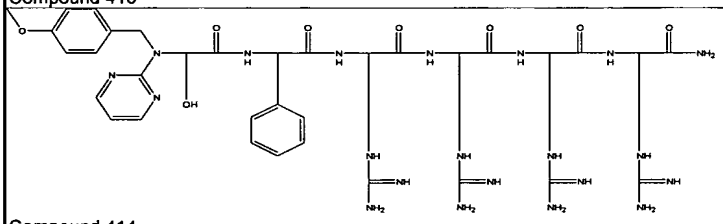
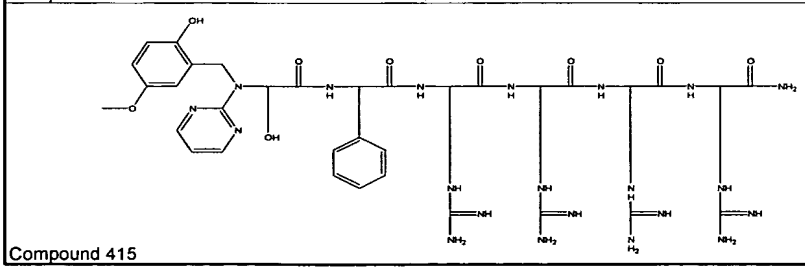
 Compound 383	C51H75N21O11	1158.2876	<10%
 Compound 384	C53H80N22O11	1201.3558	<10%
 Compound 385	C51H76N22O13	1205.301	<10%
 Compound 386	C57H79N21O11	1234.3852	<10%
 Compound 387	C53H75N21O12	1198.309	<10%

 Compound 388	C59H79N21O11	1258.4072	<10%
 Compound 389	C57H77N21O11	1232.3694	<10%
 Compound 390	C57H77N21O11	1232.3694	<10%
 Compound 391	C50H75N21O12	1162.276	<10%
 Compound 392	C50H75N21O13	1178.2754	<10%

 Compound 393	C43H73N21O7	996.1862	<10%
 Compound 394	C45H77N21O7	1024.2398	<10%
 Compound 395	C46H79N21O7	1038.2666	<10%
 Compound 396	C47H73N21O8	1060.2296	<10%
 Compound 397	C49H77N21O9	1104.2826	<10%
 Compound 398	C49H78N22O8	1103.2978	<10%

 Compound 399	C49H78N22O7	1087.2984	<10%
 Compound 400	C47H74N22O9	1091.2436	<10%
 Compound 401	C47H72N22O9	1089.2278	<10%
 Compound 402	C47H72N22O10	1105.2272	<10%
 Compound 403	C47H71Cl2N21O7	1113.1204	<10%
 Compound 404	C47H73N23O11	1136.2412	<10%

 Compound 405	C47H73N23O12	1152.2406	<10%
 Compound 406	C49H75N21O7	1070.268	<10%
 Compound 407	C51H80N22O7	1113.3362	<10%
 Compound 408	C49H76N22O9	1117.2814	<10%
 Compound 409	C55H79N21O7	1146.3656	<10%

 Compound 410	C51H75N21O8	1110.2894	<10%
 Compound 411	C57H79N21O7	1170.3876	<10%
 Compound 412	C55H77N21O7	1144.3498	<10%
 Compound 413	C55H77N21O7	1144.3498	<10%
 Compound 414	C48H75N21O8	1074.2564	<10%
 Compound 415	C48H75N21O9	1090.2558	<10%

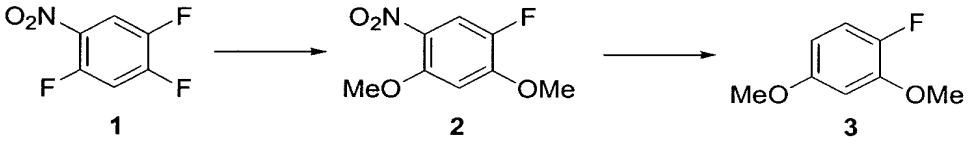
EXAMPLE II

1. Materials, Methods, and Detailed Synthesis of Compounds

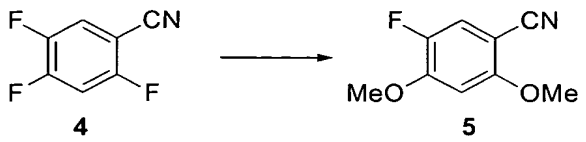
[00106] *General Procedures.* For all compounds, ^{1}H NMR, ^{13}C NMR and ^{19}F NMR spectra were recorded on a Bruker AC-300 (300 MHz) NMR spectrometer. Proton and carbon chemical shifts are reported relative to TMS using either TMS or the residue solvent signal as internal standard. Carbon NMRs are proton-decoupled, and coupling constants to fluorine are not reported. Fluorine chemical shifts are reported relative to CF_3COOH (-78.5 ppm) as an external standard. Electrospray mass spectra (ESI) data were obtained from the Laboratory for Macromolecular Analysis and Proteomics of the Albert Einstein College of Medicine. All moisture and air sensitive experiments were performed under a positive pressure of N_2 or Ar in oven dried glassware. Reagent and anhydrous solvents were bought from Aldrich Chemical Company and used without further purification.

[00107] The numbering of compounds in Example II is independent of the numbering used in Examples I and III.

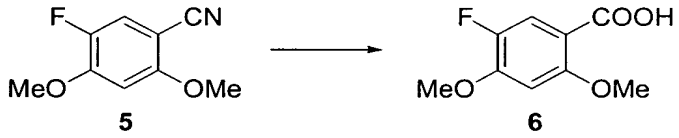
[00108] *Synthesis of 1,3-Dimethoxy-4-fluorobenzene* (**3**). Synthesis was carried out using the procedures describe in Wei-Chuan Sun et al.[52]



[00109] *Synthesis of 5-Fluoro-2,4-dimethoxy-benzonitrile* (**5**). Sodium methoxide (25% wt in MeOH, 32 mL) was added dropwise to a solution of 2,4,5-trifluorobenzonitrile (10g, 63.7 mmol) in MeOH (160 mL) under nitrogen at 0 °C. The resulting reaction mixture was stirred at reflux for 48 h. The reaction was then quenched with 1 M citric acid and the MeOH was removed *in vacuo*. The residue was taken up in EtOAc, washed with brine, dried over Na_2SO_4, concentrated *in vacuo* and recrystallized from EtOAc/hexane to furnish the desired product in 95% yield (11 g). ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.25 (d, 1 H, J = 10.2 Hz), 6.52 (d, 1 H, J = 6.8 Hz), 3.97 (s, 3 H), 3.93 (s, 3 H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 159.3, 159.2, 152.8, 152.6, 147.6, 144.4, 119.8, 119.5, 115.8, 115.7, 97.4, 97.3, 92.1, 92.0, 56.6, 56.5; ^{19}F NMR (282 MHz, $CDCl_3$) δ -143.4 (dd, 1 F); mass spectrum, calculated for $C_9H_8FNO_2$ (MH^+) 182.1, Found 182.1.

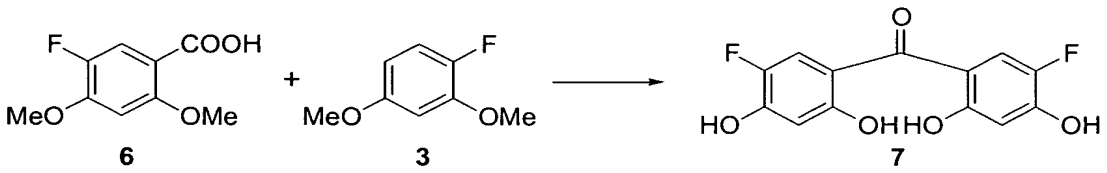


[00110] *5-Fluoro-2,4-dimethoxy-benzoic acid* (**6**). A solution of 3.1 g (17.2 mmol) of 2,4-dimethoxy-5-fluorobenzonitrile and 100 mL of 6N aqueous sodium hydroxide in 200 mL of methanol was stirred at reflux overnight, cooled to 0 °C, acidified to pH 1 - 2 with 6N aqueous hydrochloric acid, and partitioned between ethyl acetate and brine. The organic extract was dried over Na_2SO_4, and the solvents were removed *in vacuo* to give crude 2,4-dimethoxy-5-fluorobenzoic acid as a light-yellow solid. Recrystallization from ethyl acetate and hexane afforded 3.2 g (93%) of **6** as a white solid. ^{1}H NMR (300 MHz, DMSO-d_6) δ 12.4 (s, 1 H), 7.49 (d, 1 H, J = 12 Hz), 6.83 (d, 1 H, J = 7.2 Hz), 3.92 (s, 3 H), 3.84 (s, 3 H); ^{13}C NMR (75 MHz, DMSO-d_6) δ 165.5, 165.5, 156.7, 151.1, 151.0, 146.3, 143.2, 117.9, 117.6, 111.2, 111.1, 99.3, 56.6, 56.4; ^{19}F NMR (282 MHz, DMSO-d_6) δ-146.2 (dd, 1F); mass spectrum, calculated for $C_9H_9FO_4$ (MH^+) 201.1, Found 200.9.

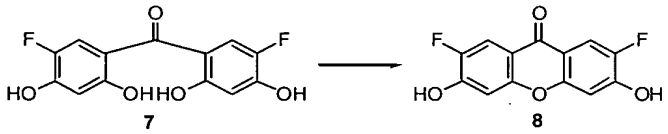


[00111] *5,5'-difluoro-2,2',4,4'-tetrahydroxybenzophenone* (**7**). 2,4-dimethoxy-5-fluorobenzoic acid **6** (5.4 g, 27 mmol) in dry CH_2Cl_2 (100 mL) was treated with 10.6 mL of oxalyl chloride under an Ar atmosphere and stirred at room temperature overnight. The excess reagent and solvent were removed under reduced pressure. The residue, 2,4-dimethoxy-5-fluorobenzoyl chloride, was redissolved in anhydrous CH_2Cl_2 (125 mL). 1,3-dimethoxy-4-fluorobenzene **3** (3.29 mL, 26 mmol) and $AlCl_3$ (10.8 g, 81 mmol) were added at 0 °C. After stirring at 0 °C for 1 h, the reaction mixture was allowed to warm to room temperature for 67 h, the mixture was then hydrolyzed with 1M HCl (300 mL) at 0 °C and extracted with EtOAc. The combined organic layers were washed with brine, dried over Na_2SO_4 and concentrated *in vacuo*. The crude product was purified by flash column chromatography, eluting with 30% - 40% EtOAc in hexane to yield **7** (3.3 g, 44%) as a yellow solid. ^{1}H NMR (300 MHz, MeOH-d_4) 7.14 (d, 2 H, J = 11.5 Hz), 6.46 (d, 2 H, J = 7.4 Hz); ^{13}C NMR (75 MHz, MeOH-d_4) δ 199.1, 159.0, 152.9, 152.7, 147.7, 144.5,

119.2, 118.9, 114.2, 114.1, 106.1, 106.0; ^{19}F NMR (282 MHz, MeOH-d_4) δ–149.8 (dd, 2F).

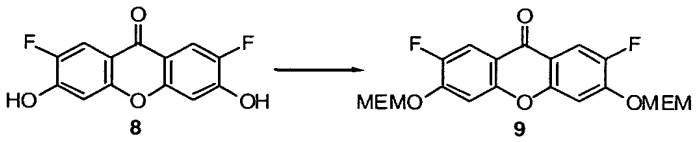


[00112] *2,7-Difluoro-3,6-dihydroxy-xanthen-9-one* (**8**). Compound **7** (0.75 g, 2.66 mmol) was heated in a sealed tube with H_2O (30 mL) at 200 - 220 0 °C for 2 - 3 h. On cooling, 2,7-difluoro-3,6-dihydroxyxanthone **8** precipitated out. The product was collected by filtration and washed with H_2O to give **8** (0.58 g, 83%) as a light yellow solid. ^{1}H NMR (300 MHz, DMSO-d_6) δ 11.4 (s, 2 H), 7.67 (d, 2 H, J = 10.9 Hz), 6.96 (d, 2 H, J = 7.1 Hz); ^{13}C NMR (75 MHz, DMSO-d_6) δ 173.1, 153.1, 152.0, 151.8, 150.4, 147.2, 112.6, 112.5, 111.1, 110.8, 104.7, 104.6; ^{19}F NMR (282 MHz, DMSO-d_6) δ-139.0 (dd, 2F); mass spectrum, calculated for $C_{13}H_6F_2O_4$ (MH^+) 265.0, Found 264.9.

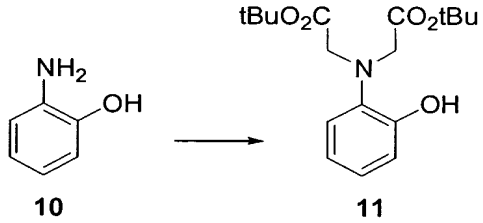


[00113] *2,7-Difluoro-3,6-bis-(2-methoxyethoxymethoxy)-xanthen-9-one* (**9**). A cloudy solution of **8** (2.4 g, 9.1 mmol) in dry THF (50 mL) was treated with sodium hydride (1.1 g, 45.8 mmol) at 0°C under an Ar atmosphere. The mixture was stirred at 0°C for 30 min and then 2-methoxyethoxymethyl chloride (5.2 mL, 45.8 mmol) was added. The mixture was stirred at 0°C for an additional 30 min and then warmed to room temperature overnight. The reaction mixture was cooled to 0°C, quenched with 1M citric acid (50mL), and then extracted with EtOAc. The combined organic layers were washed with brine, dried over Na_2SO_4 and concentrated *in vacuo*. The crude product was purified by flash column chromatography, eluting with CH_2Cl_2/hexane/EtOAc = 1:1:0.4 to yield 2.6 g (65%) of **9** as a white solid. R_f= 0.47 (in CH_2Cl_2/hexane/EtOAc = 1:1:0.4); ^{1}H NMR (300 MHz, $CDCl_3$) δ7.91 (d, 2 H, J = 10.6 Hz), 7.28 (d, 2 H, J = 6.5 Hz), 5.46 (s, 4 H), 3.91 (m, 4 H), 3.60 (m, 4 H), 3.39 (s, 6 H); ^{13}C NMR (75 MHz, $CDCl_3$) δ174.4, 153.3, 153.2, 151.4, 151.0, 150.9, 148.2, 115.2, 115.1, 111.8, 111.6, 104.8, 94.2, 71.3, 68.6, 59.0; ^{19}F

NMR (282 MHz, $CDCl_3$) δ-137.0 (dd, 2 F); mass spectrum, calculated for $C_{21}H_{22}F_2O_8$ (MH^+) 441.1, Found 441.0.

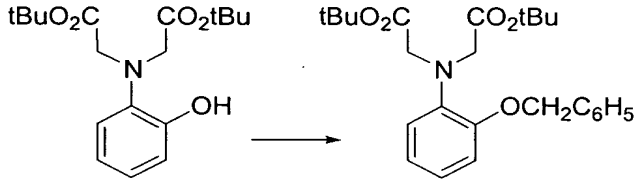


[00114] *[tert-Butoxycarbonylmethyl-(2-hydroxy-phenyl)-amino]-acetic acid tert-butyl ester* (**11**). A solution of 2-aminophenol (**10**) (5.0 g, 45.8 mmol) and NaI (3.44 g, 23 mmol) in dry CH_3CN (150 mL) was treated with 1,8-bis(dimethylamino) naphthalene (21.6 g, 100.8 mmol) followed by t-butyl bromoacetate (14.2 mL, 96.2 mmol) under an Ar atmosphere and stirred at reflux overnight. After the mixture was cooled to room temperature, the precipitated salts were filtered off and washed with additional EtOAc. The combined organic layers were washed with brine, dried over Na_2SO_4 and concentrated *in vacuo*. The crude product was purified by flash column chromatography, eluting with CH_2Cl_2/hexane/EtOAc = 1:4:0.2 to yield 12.3 g (80%) of the desired product (**11**) as a light yellow oil. R_f = 0.3 (in CH_2Cl_2/hexane/EtOAc = 1:4:0.2); ^1H NMR (300 MHz, $CDCl_3$) δ 7.29 (dd, 7.9 and 1.5 Hz, 1 H), 7.07 (m, 1 H), 6.94 (dd, 8.1 and 1.5 Hz, 1 H), 6.79 (m, 1 H), 5.30 (s, 1 H), 3.74 (s, 4 H), 1.46 (s, 18 H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 171.2, 153.6, 137.4, 127.3, 126.0, 119.9, 115.6, 81.9, 57.0, 28.0; mass spectrum, calculated for $C_{18}H_{27}NO_5$ (MH^+) 338.2, Found 337.9.



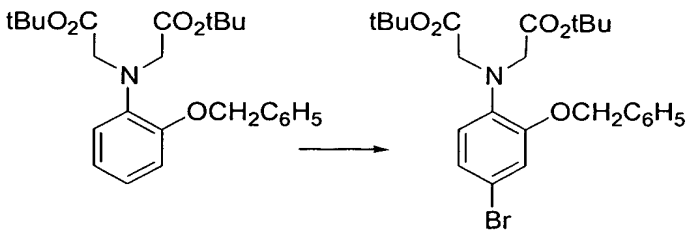
[00115] *[(2-Benzyloxy-phenyl)-tert-butoxycarbonylmethyl-amino]-acetic acid tert-butyl ester* (**12**). A solution of [*tert*-Butoxycarbonylmethyl-(2-hydroxy-phenyl)-amino]-acetic acid *tert*-butyl ester (**11**) (7.4 g, 21.9 mmol) in dry THF (50 mL) was treated with sodium hydride (0.81 g, 33.8 mmol) at 0°C under an Ar atmosphere. After stirring at 0°C for 30 min, benzyl bromide (3.9 mL, 32.7 mmol) was added. The mixture was stirred at 0°C for another 30 min and then warmed to room temperature overnight. The reaction mixture was quenched with H_2O and extracted with EtOAc. The combined organic layers were

washed with brine, dried over Na_2SO_4 and concentrated *in vacuo*. The crude product was purified by flash column chromatography, eluting with CH_2Cl_2/hexane/EtOAc = 1:4:0.2 to yield 6.3 g (67%) of the desired product **12** as a colorless oil. R_f = 0.33 (in CH_2Cl_2/hexane/EtOAc = 1:4:0.2); ^1H NMR (300 MHz, $CDCl_3$) δ 7.45 - 7.28 (m, 5 H), 6.84 (m, 4 H), 5.12 (s, 2 H), 4.08 (s, 4 H), 1.40 (s, 18 H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 170.5, 150.5, 139.6, 137.5, 128.4, 127.6, 127.2, 121.9, 121.4, 119.4, 114.7, 81.0, 70.9, 54.6, 28.0; mass spectrum, calculated for $C_{25}H_{33}NO_5$ (MH^+) 428.2, Found 427.9.



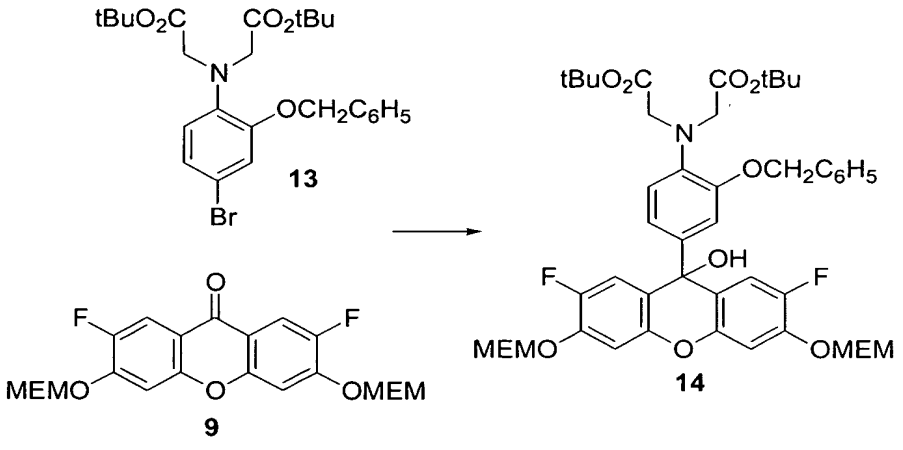
11 **12**

[00116] [(2-Benzyloxy-4-bromo-phenyl)-*tert*-butoxycarbonylmethyl-amino]-acetic acid *tert*-butyl ester (**13**). To a −78 °C solution of [(2-Benzyloxy-phenyl)-*tert*-butoxycarbonylmethyl-amino]-acetic acid *tert*-butyl ester (6.3 g, 14.7 mmol) in CH_2Cl_2 (100 mL) was added pyridine (1.8 mL, 22.1 mmol) followed by bromine (0.91 mL, 17.6 mmol) under an Ar atmosphere. After 30 min, the mixture was allowed to warm to room temperature and then washed with water, 5% sodium bicarbonate, and brine. The organic layer was dried over Na_2SO_4 and concentrated *in vacuo*. The crude product was purified by flash column chromatography, eluting with CH_2Cl_2/hexane/EtOAc = 1:4:0.2 to yield 5.9 g (79 %) of **13** as a white solid. R_f = 0.43 (in CH_2Cl_2/hexane/EtOAc = 1:4:0.2); ^1H NMR (300 MHz, $CDCl_3$) δ 7.45 - 7.26 (m, 5 H), 6.99 (m, 2 H), 6.73 (m, 1 H), 5.09 (s, 2 H), 4.04 (s, 4 H), 1.41 (s, 18 H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 170.1, 151.1, 138.7, 136.6, 128.6, 127.9, 127.4, 124.1, 120.5, 117.6, 113.7, 81.2, 71.2, 54.5, 28.0; mass spectrum, calculated for $C_{25}H_{32}BrNO_5$ (MH^+) 506.2, Found 505.8.

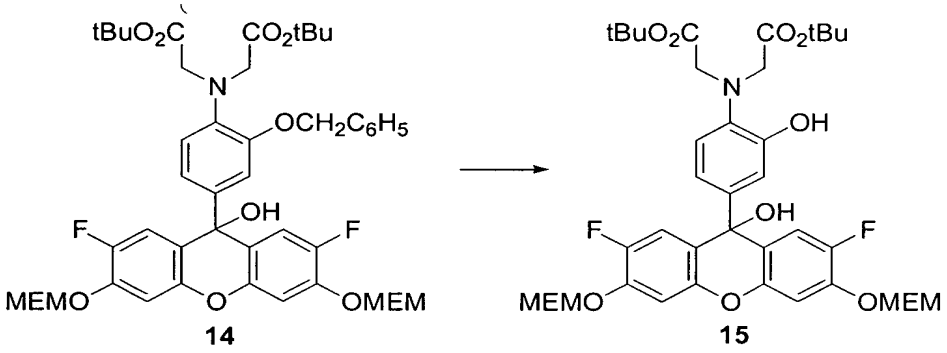


12 **13**

[00117] *({2-Benzyloxy-4-[2,7-difluoro-9-hydroxy-3,6-bis-(2-methoxyethoxymethoxy)-9H xanthen-9-yl]-phenyl}-tert-butoxycarbonylmethyl-amino)-acetic acid tert-butyl ester* (**14**). The bromide **13** (807 mg, 1.59 mmol) was dissolved in 20 mL THF/2-methyltetrahydrofuran (1:1) and cooled to –105 °C in a liquid N_2/diethyl ether bath. After stirring at –105 °C for 10 min, 4.5 mL of tert-butyllithium (1.1 M in pentanes) was added dropwise. Stirring was continued for another 15 min. 2,7-difluoro-3,6-bis-(2-methoxy-ethoxymethoxy)-xanthen-9-one **9** (912 mg, 2.07 mmol) dissolved in THF (10 mL) was added dropwise to the reaction mixture. The mixture was stirred at -105°C for 30 min, the cooling bath removed, and the reaction solution was then stirred for an additional 15 min. The resulting mixture was added to 150 mL NH_4Cl(sat) and extracted twice with EtOAc. The combined organic layers were washed with brine, dried over Na_2SO_4 and concentrated *in vacuo*. The crude product was purified by flash column chromatography, eluting with 50% EtOAc in hexane (with 0.1% Et_3N) to yield 956 mg (69%) of **14** as a orange-red oil. R_f = 0.33 (in 50% EtOAc/hexane); ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.32 - 7.27 (m, 5 H), 6.97 (d, 2 H, J = 6.98 Hz), 6.86 (m, 3 H), 6.72 (m, 2 H), 5.30 (s, 4 H), 5.05 (s, 2 H), 4.02 (s, 4 H), 3.85 (m, 4 H), 3.56 (m, 4 H), 3.36 (s, 6 H), 1.38 (s, 18 H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 170.5, 150.8, 149.3, 147.6, 145.6, 145.5, 145.4, 145.2, 140.5, 138.4, 136.9, 128.3, 127.7, 127.5, 119.9, 119.8, 119.3, 118.4, 115.2, 114.9, 113.0, 104.9, 94.5, 81.0, 71.4, 70.6, 69.7, 68.1, 58.9, 54.6, 28.0; ^{19}F NMR (282 MHz, $CDCl_3$) δ -139.0 (dd, 2 F); mass spectrum, calculated for $C_{46}H_{55}F_2NO_{13}$ (MH^+) 868.4, Found 867.8.

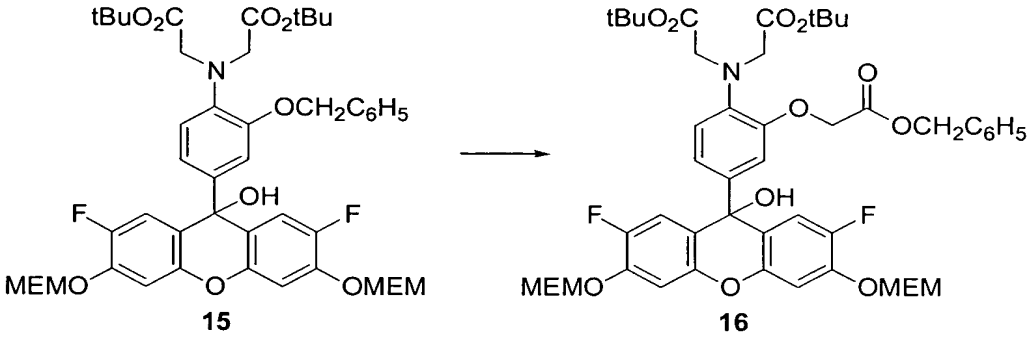


[00118] *(tert-Butoxycarbonylmethyl-{4-[2,7-difluoro-9-hydroxy-3,6-bis-(2-methoxyethoxymethoxy)-9H-xanthen-9-yl]-2-hydroxy-phenyl}-amino)-acetic acid tert-butyl ester* (**15**). To a solution of **14** (956 mg, 1.1 mmol) in 95% ethanol (25 mL) and EtOAc (25 mL) was added 10% Pd/C (130 mg). The resulting mixture was stirred at room temperature overnight under H_2 (1 atm). The catalyst was then removed by filtration and, after evaporation to dryness, the crude product was purified by flash column chromatography, eluting with 40% EtOAc in hexane (with 0.1% Et_3N) to yield 671 mg (78%) of **15** as a light yellow oil. R_f= 0.3 (in 40% EtOAc/hexane); ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.1 (s, 1 H), 7.18 (d, 1 H, J = 8.0 Hz), 6.95 (d, 2 H, J = 7.2 Hz), 6.69 (m, 3 H), 6.57 (m, 1 H), 5.28 (s, 4 H), 4.94 (s, 1 H), 3.85 (m, 4 H), 3.69 (s, 4 H), 3.57 (m, 4 H), 3.38 (s, 6 H), 1.41 (s, 18 H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 171.2, 153.8, 150.6, 147.4, 146.5, 144.8, 144.4, 144.2, 136.3, 126.3, 119.6, 116.7, 116.6, 116.1, 115.8, 115.5, 105.9, 94.6, 81.9, 71.4, 68.0, 59.0, 56.9, 43.1, 27.9; ^{19}F NMR (282 MHz, $CDCl_3$) δ -140.4 (dd, 2 F); mass spectrum, calculated for $C_{46}H_{55}F_2NO_{13}$ (M-17) 761.3, Found 761.7.

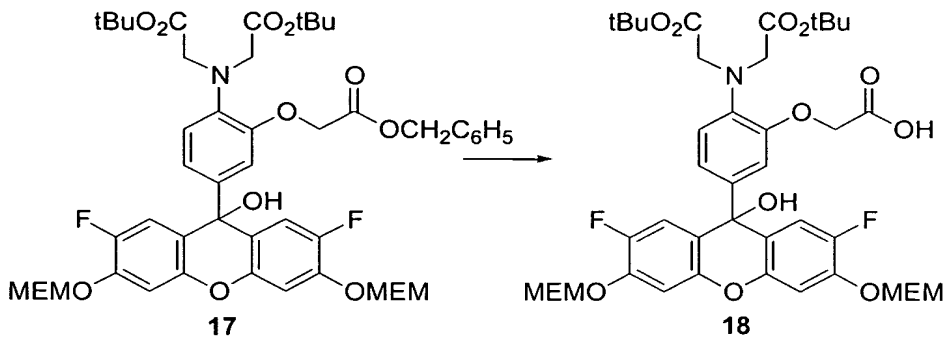


[00119] *({2-Benzyloxycarbonylmethoxy-4-[2,7-difluoro-9-hydroxy-3,6-bis-(2-methoxyethoxymethoxy)-9H-xanthen-9-yl]-phenyl}-tert-butoxycarbonylmethyl-amino)-acetic acid tert-butyl ester* (**16**). A solution of **15** (497 mg, 0.64 mmol) in DMF (10 mL) was treated with sodium hydride (18.5 mg, 0.77 mmol) at 0°C under an Ar atmosphere. After stirring at 0°C for 30 min, benzyl 2-bromoacetate (0.15 mL, 0.96 mmol) was added. The mixture was stirred at 0°C for another 30 min and then warmed to room temperature overnight. The solvent was evaporated to dryness and extracted with EtOAc/H_2O. The combined organic layers were washed with brine, dried over Na_2SO_4 and concentrated *in vacuo*. The crude product was purified by flash column chromatography, eluting with 40% EtOAc in Hexane (with 0.1% Et_3N) to yield 453 mg (76%) of **16** as a light yellow oil. R_f= 0.28 (in 40% EtOAc/hexane); ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.32 (m, 5 H), 6.96

(d, 2 H, J = 7.2 Hz), 6.86 (d, 1 H, J = 8.4 Hz), 6.72 (dd, 1 H, J = 7.8 and 1.5 Hz), 6.59 (m, 3 H), 5.28 (s, 4 H), 5.15 (s, 2 H), 4.89 (s, 1 H), 4.64 (s, 2 H), 4.01 (s, 4 H), 3.86 (m, 4 H), 3.58 (m, 4 H), 3.39 (s, 6 H), 1.39 (s, 18 H); ^{13}C NMR (75 MHz, $CDCl_3$) δ170.3, 168.8, 150.7, 149.9, 147.5, 146.6, 146.5, 144.4, 144.3, 139.1, 138.9, 135.3, 128.6, 128.4, 128.3, 122.9, 120.0, 117.0, 116.9, 116.2, 116.1, 115.8, 105.9, 94.7, 81.2, 71.5, 68.1, 66.7, 66.6, 59.0, 54.5, 42.6, 28.0; ^{19}F NMR (282 MHz, $CDCl_3$) δ -140.2 (dd, 2 F); mass spectrum, calculated for $C_{46}H_{55}F_2NO_{13}$ (M-17) 909.4, Found 910.1.



[00120] *{2-(Bis-tert-butoxycarbonylmethyl-amino)-5-[2,7-difluoro-9-hydroxy 3,6-bis-(2-methoxy-ethoxymethoxy)-9H-xanthen-9-yl]-phenoxy}-acetic acid* (**18**). To a solution of **17** (743 mg, 0.80 mmol) in 95% ethanol (25 mL) and EtOAc (25 mL) was added 10% Pd/C (135 mg). The resulting mixture was stirred at room temperature overnight under H_2 (1 atm). The catalyst was then removed by filtration and, after evaporation to dryness, the crude product was purified by flash column chromatography, eluting with 10% MeOH in CH_2Cl_2 to yield 595 mg (89%) of **18** as a red foaming solid. R_f = 0.43 (in 10% MeOH/CH_2Cl_2); ^{1}H NMR (300 MHz, $CDCl_3$) δ 10.0 (br, 1 H), 6.93 (d, 2 H, J = 7.2 Hz), 6.86 (d, 1 H, J = 8.2 Hz), 6.71 (dd, 1 H, J = 8.2 and 1.3 Hz), 6.62 (m, 3 H), 5.24 (s, 4 H), 4.93 (s, 1 H), 4.53 (s, 2 H), 3.91 (s, 4 H), 3.83 (m, 4 H), 3.56 (m, 4 H), 3.35 (s, 6 H), 1.34 (s, 18 H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 171.0, 170.5, 150.6, 150.5, 147.4, 146.4, 146.3, 144.4, 144.2, 140.8, 138.7, 122.3, 120.7, 116.6, 116.5, 115.9, 115.6, 115.4, 105.9, 94.6, 81.7, 71.3, 67.9, 67.3, 58.8, 55.0, 42.7, 27.8; ^{19}F NMR (282 MHz, $CDCl_3$) δ -140.0 (dd, 2 F); mass spectrum, calculated for $C_{46}H_{55}F_2NO_{13}$ (M-17) 819.3, Found 819.8.



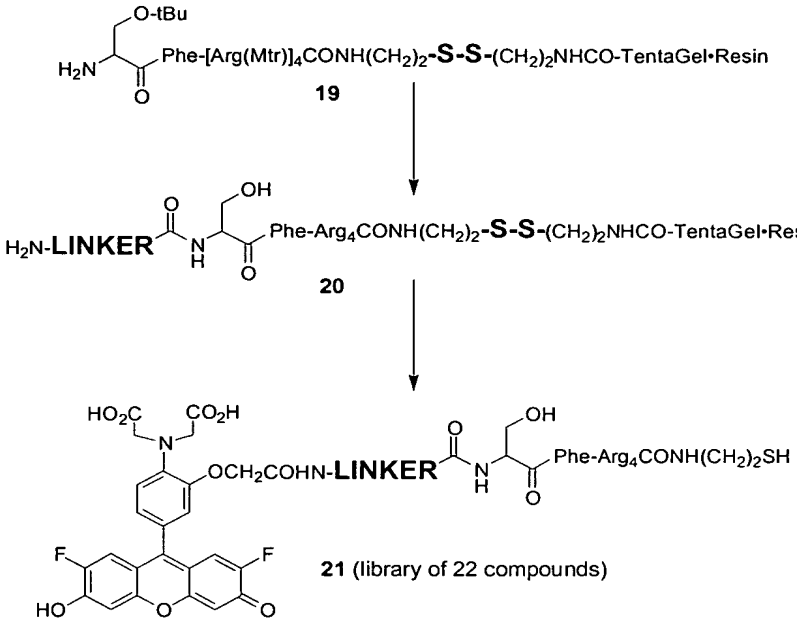
[00121] *H_2N-Ser(tBu)-Phe-[Arg(Mtr)]$_4$-cystamine-Tentagel Resin* (**19**). Cystamine dihydrochloride (10 eq, 2.25 g) was added to a mixture of TentaGel S COOH resin (90 μm, 5 g, 0.2 mmol/g), BOP (1.2 eq, 0.53 g), HOBt (1.2 eq, 0.184 g), N-methylmorpholine (NMM) (30 eq, 3.3 mL) in 20 mL DMF and subsequently shaken overnight at ambient temperature. The free amine substitution level was determined as 0.01 mmol/g. This low substitution level is ideal for our purposes since this not only ensures a higher coupling yield but, in addition, larger quantities of resin (with greater weight accuracy) can be subsequently introduced into the 96-well plates. The peptide NH_2-Ser(tBu)-Phe-[Arg(Mtr)]$_4$ was synthesized on the cystamine-substituted TentaGel resin using an Fmoc solid phase peptide synthesis protocol.

[00122] *General protocol for the preparation of the fluorophore-peptide conjugates* (**21** (Scheme 3) and **23**). Both compound **23** and the corresponding linker-based library **21** (23 compounds) were prepared using the same protocol, with the exception that the library members were synthesized in a solvent resistant 96-well filter plate. 10 mg of the peptide-TentaGel resin **19** was introduced into 23 individual wells of a 96-well filter plate. In addition, each well contained a Fmoc-linker-COOH (10 eq), *O*-benzotriazol-1-yl-*N,N,N',N'*-tetramethyluronium hexafluorophosphate (10 eq), 1-hydroxybenztriazole (10 eq), and N-methylmorpholine (20 eq) in 100 μL DMF. A total of 22 different Fmoc-linker-COOH linkers were employed. One additional well was reserved for the species that does not contain a linker. The plate was shaken overnight and then each well was subjected to a series of wash steps (3 × 200 μL DMF, 3 × 200 μL water, 3 × 200 μL DMF, 3 × 200 μL CH_2Cl_2, 2 × 200 μL MeOH, 2 × 200 μL 50 mM Tris pH 7.5). The N-terminal Fmoc protecting group was removed via double exposure to 30% piperidine in DMF for 30 min and then each well was subjected to a series of wash steps (3 × 200 μL DMF, 3 × 200 μL water, 3 × 200 μL DMF, 3 × 200 μL CH_2Cl_2, 2 × 200 μL MeOH, 2 × 200 μL 50

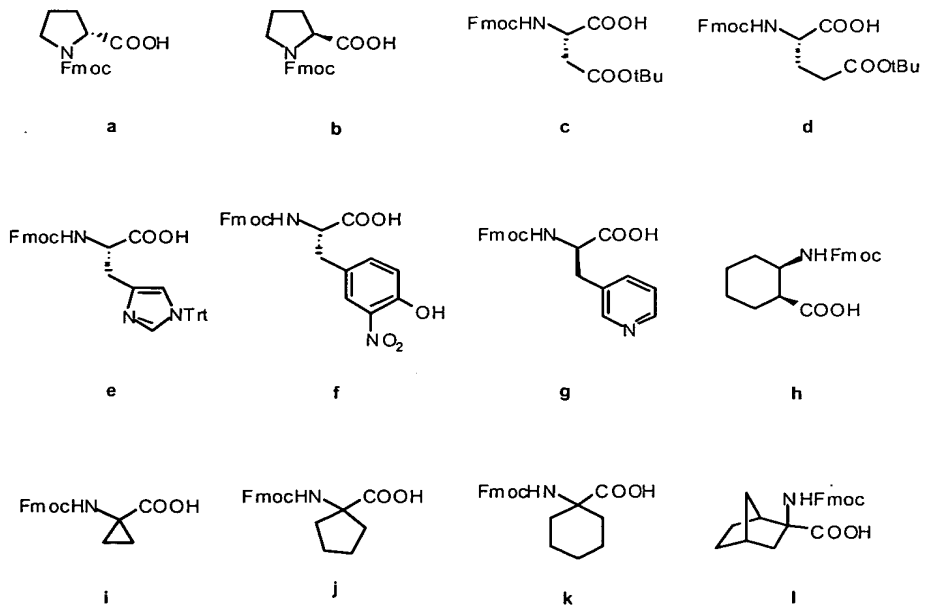
mM Tris pH 7.5). The side chain protecting groups on the peptide were removed via treatment with 95% trifluoroacetic acid/2.5% thioanisole/2.5% H_2O at room temperature overnight to furnish **20**. Each well was subsequently subjected to a series of wash steps (3 × 200 μL DMF, 3 × 200 μL water, 3 × 200 μL DMF, 3 × 200 μL CH_2Cl_2, 2 × 200 μL MeOH, 2 × 200 μL 50 mM Tris pH 7.5). {2-(Bis-tert-butoxycarbonylmethyl-amino)-5-[2,7-difluoro-9-hydroxy 3,6-bis-(2-methoxy-ethoxymethoxy)-9H-xanthen-9-yl]-phenoxy}-acetic acid (**18**) (5 eq) was coupled to the peptide-TentaGel resin **20** under standard conditions [*O*-benzotriazol-1-yl-*N,N,N',N'*-tetramethyluronium hexafluorophosphate (5 eq), 1-hydroxybenztriazole (5 eq), and N-methylmorpholine (10 eq) in 100 μL DMF]. The attached xanthene was then deprotected and fully aromatized via exposure to 95% trifluoroacteic acid/5% H_2O. The fluorophore-peptide conjugates were cleaved from the disulfide-containing resin with 10 mM dithiothreitol (DTT) in Tris buffer (1 × 200 μL for 1 Hr; 2 × 150 μL for 1 hr each) and filtered into a receiving set of 96-well plates using a vacuum manifold to furnish the library **21**, shown below.

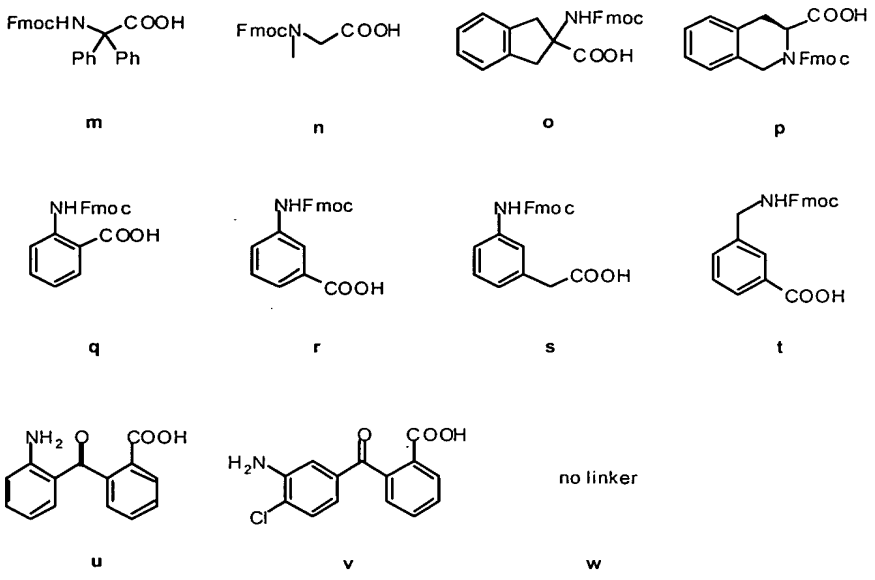
[00123] Mass spectrum, calculated for compound **21a** 1544.7, Found 1624.0 (M + 79.9); calculated for **21b** 1544.7, Found 1544.0; calculated for **21c** 1562.6, Found 1562.0; calculated for **21d** 1576.7, Found 1576.0; calculated for **21e** 1584.7.7, Found 1584.0; calculated for **21f** 1655.7, Found 1657.0 (M + 2); calculated for **21g** 1595.7, Found 1596.0; calculated for **21h** 1572.7, Found 1572.0; calculated for **21i** 1530.6, Found 1530.0;calculated for **21j** 1558.7, Found 1558.0; calculated for **21k** 1572.7, Found 1574.0; calculated for **21l** 1584.0, Found 1584.0 (M + 2); calculated for **21m** 1518.0, Found 1518.0; calculated for **21n** 1517.7, Found 1518.0; calculated for **21o** 1606.7, Found 1607.0; calculated for **21p** 1606.7, Found 1607.0 (M + 2); calculated for **21q** 1566.7, Found no identifiable mass ion; calculated for **21r** 1566.7, Found 1566.0; calculated for **21s** 1580.7, Found 1581.0; calculated for **21t** 1580.7, Found 1581.0; calculated for **21u** 1670.7, Found no identifiable mass ion; calculated for **21v** 1796.2, Found no identifiable mass ion; calculated for **21w** 1447.5, Found 1447.0.

Scheme 3. Synthesis of library **21**.



[00124] Starting materials for the 22 linker monomers (plus compound w (no linker)) employed in the preparation of library **21** are shown below:





[00125] *Protein kinase C assay.* The kinase-catalyzed reaction was performed in triplicate at 30 °C and initiated by addition of ATP to a solution of PKCα and fluorophore-appended peptide substrate **23** (except in the case of the library, where the assays were performed in a 96 well plate using a fluorescence plate reader). Final conditions: 62.5 mM HEPES, 3 mM $MgCl_2$, 0.3 mM $CaCl_2$, 0.1 mM EGTA, 1 mM DTT, 0.5 μg/mL phosphatidylserine, 0.1 μg/mL diacylglycerol, 1 mM ATP, and 13 nM PKCα (pH 7.4). After the addition of ATP, the solution was gently mixed and the time-dependent change in fluorescent intensity ($\lambda_{excitation}$ = 494 nm; $\lambda_{emission}$ = 521 nm) continuously monitored with a Photon Technology QM-1 spectrofluorimeter.

[00126] *Ca^{2+} titration assay.* The Ca^{2+} Calibration Kit #2 (purchased from Molecular Probes) was employed for these studies. The fluorescent peptide was dissolved in two buffers, one without (SOLUTION A: 10 mM MOPs, 100 mM KCl, 10 mM EGTA, pH 7.2) and one with (SOLUTION B: 10 mM MOPs, 100 mM KCl, 10 mM CaEGTA, pH 7.2) Ca^{2+}. The final concentration of the peptide in both buffers was 1 μM. Solution A was (200 μL) added to the microcuvette and the fluorescence was recorded ($\lambda_{excitation}$ = 494 nm; $\lambda_{emission}$ = 521 nm). This was the fluorescence intensity with 0 Ca^{+2}. A series of

solutions containing the following free Ca^{2+} concentrations (based on the K_d of CaEGTA at pH 7.2 = 150.3 nM) was prepared: 0.038 μM, 0.065 μM, 0.1 μM, 0.15 μM, 0.225 μM, 0.351 μM, 0.602 μM, 1.35 μM, and 39.8 μM.

2. Results and Discussion

[00127] An example of a fluorescent sensor that samples biologically relevant processes is the family of Ca^{2+} indicators (e.g. compound **22** in Scheme 4, below) developed by Tsien and his colleagues.[39-41] Formation of the Ca^{2+}-fluorophore complex, *via* coordination to the 2 iminodiacetic acid moieties, is manifested by a dramatic fluorescence change. Tsien has proposed that Ca^{2+} coordination induces a twist about the aryl amine bond, altering the coupling between the nitrogen lone pair and the aromatic ring system.[39-41]

[00128] A peptide-based species (compound **23** in Scheme 4) was designed that contains some of the structural features present in **22**. Specifically, phosphorylation of **23** should generate a M^{2+} receptor site comprised of two carboxylates and the newly introduced phosphate (**24**). Upon divalent metal ion coordination, a fluorescence change should transpire via a mechanism analogous to that described for the Ca^{2+} indicators.

[00129] This strategy is made possible by the observation that protein kinases will phosphorylate alcohol-containing residues attached to the N- or C-terminus of appropriately designed peptides,[20,24] which allows the fluorophore to be directly attached to the phosphorylatable residue (e.g. **23**). With these features in mind, the initial synthetic target was compound **18**, which contains the requisite functionality in protected form, along with a free carboxylate that can be activated and condensed with the N-terminus of the peptide H_2N-Ser-Phe-Arg-Arg-Arg-Arg-resin (SEQ ID NO:1). The latter sequence serves as a substrate for protein kinase C (PKC).[24]

[00130] The synthesis of the fluorescein precursor compound **18** is shown in Scheme 5. The xanthene half of **18** was synthesized from the xanthone precursor **7**. The latter was prepared via the Friedel-Crafts acylation of **3**[42] with **6**. The product was subsequently heated in a sealed tube to furnish the xanthone **8** and the phenol moieties then protected as 2-methoxyethoxymethyl (MEM)[43] ethers (**9**). The aromatic precursor (**13**) to the "northern" half of compound **18** was prepared in three steps from commercially available *o*-aminophenol (described above). Compound **13** contains a doubly protected iminodiacetic acid moiety and a benzylated phenol. The latter will ultimately be

debenzylated so that it can serve as the attachment site for the peptide. Compound **13** was lithiated at –105 °C and coupled to **9**, to furnish adduct **14** in 69% yield. The benzylated phenol in **14** was transformed in three steps to the desired carboxylic acid (**18**), and then coupled to H_2N-Ser(*O*-tBu)-Phe-[(Arg)Mtr]$_4$-resin (prepared via standard Fmoc solid phase peptide synthesis on the Rink resin). Finally, exposure of the resin-appended fluorophore-peptide to 95% CF_3CO_2H resulted in the simultaneous cleavage of the peptide from the resin, MEM ether deprotection, and complete aromatization of the tricyclic nucleus via loss of the tertiary hydroxyl moiety to yield **23**.

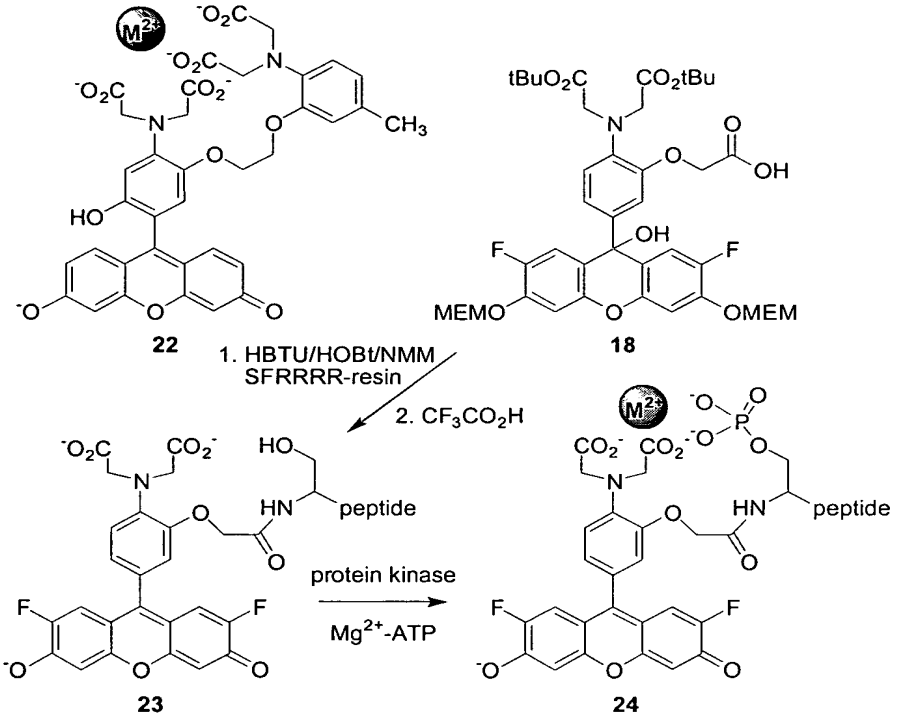
[00131] Peptide **23** serves as a substrate for the Ca^{2+}-dependent PKCα and displays a 140% increase in fluorescence intensity upon phosphorylation, nearly an order of magnitude greater than previously described protein kinase monitoring systems.[6-10,38] In addition, the difluorofluorescein moiety in **23** is an extremely bright fluorophore (ε = 78,000 cm^{-1} M^{-1} and Φ = 0.60) and thus, is easily an order of magnitude more sensitive than fluorophores that have been previously used to observe protein kinase activity.[42] Although the K_m value (26.5 μM) for the PKCα-catalyzed phosphorylation of **23** is quite good, the corresponding V_{max} (0.32 μmol/min-mg) is an order of magnitude less than ideal. The large, negatively charged fluorophore, which is positioned adjacent to the site of phosphorylation, might interfere with the ability of the PKC active site to accommodate the serine moiety.

[00132] The possibility of fluorophore-mediated disruption of the kinase-catalyzed reaction was addressed by preparing a small library of 22 derivatives of **23** using the synthetic strategy outlined in Scheme 3. A series of turn-promoting/metal chelating LINKERs was inserted between the peptide and the fluorophore (**21**), which might allow the serine moiety to be more optimally accommodated within the active site. Following phosphorylation, the turn-inducing/chelating ability of the LINKER should enable the iminodiacetic acid carboxylates to assume a position that promotes metal coordination. The library of 22 compounds was prepared on a cystamine-derivatized TentaGel resin,[44] which contains a disulfide bridge between the peptide and the resin (**19**). The side chain protected peptide **19** was split into 22 portions of 10 mg each and added to a solvent resistant multiwell filter plate. 22 Fmoc-amino acids ("LINKER"s, see above) were added to individual wells and condensed with **19**. The Fmoc group was removed (**20**) and the product coupled to compound **18**. 95% CF_3CO_2H was subsequently employed to simultaneously deprotect the phenol and carboxylic acid moieties and transform the

xanthene nucleus into the fluorescein derivative. Finally, all 22 compounds were cleaved from the Tentagel resin with PKC assay buffer, which contains dithiothreitol. The library members (**21**) (see above) were filtered into a receiving plate and then assayed under standard conditions with monitoring for both the magnitude and rate of fluorescence change. Several fluorophore-LINKER-peptide analogs were identified that display promising enzymological and photophysical properties (Table 4). N-methyl glycine serves as the LINKER in the lead protein kinase substrate. Phosphorylation of the latter generates a 264% enhancement in fluorescence intensity and proceeds with a V_{max} of 8.5 μmol/min-mg and a K_m of 20.5 μM. Indeed, the V_{max} is more than an order of magnitude greater than that displayed by compound **23**, which lacks a LINKER residue between the fluorophore and the peptide.

[00133] Saturating [Ca^{2+}] produces 1.2- and 2.0-fold fluorescence enhancements in **23** and **21** (N-Me Gly), respectively. By contrast, enhancements of 5- and 23-fold were observed with the chemically synthesized *phosphorylated* analogs of **23** and **21**, respectively. However, the large metal-induced fluorescence change in the phosphorylated species appears to be partly offset by a reduction in the inherent (i.e. metal-free) fluorescence of the phosphopeptides.

Scheme 4



Scheme 5. Synthesis of fluorescein precursor **18**.

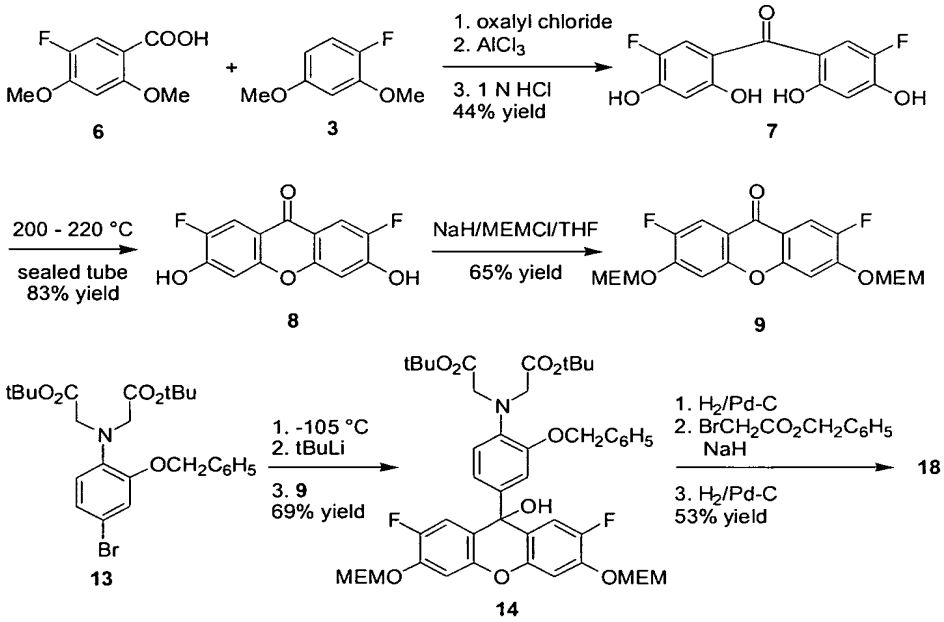
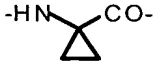
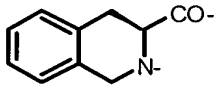


Table 4. V_{max}, K_m, and fluorescence change associated with the PKC-catalyzed phosphorylation of peptides **21** (5 different linkers), **23**, and AcSFRRRRK (SEQ ID NO:2).

LINKER	V_{max} (μmol/min -mg)	K_m (μM)	% Change Fluorescence
L-proline	1.9	63.0	150%
D-proline	1.0	23.5	156%
N-Me glycine	8.5	20.5	264%
a	1.7	25.0	164%
b	2.2	24.9	157%
Peptide **23**	0.32	26.5	140%
AcSFRRRRK	24	10	-

a 

b 

EXAMPLE III – Light-Activated ("caged") Probe of Protein Kinase Activity

1. Materials, Methods, and Detailed Synthesis of Compounds

[00134] Chemicals and solvents were purchased from Fisher, Sigma, and Aldrich, except for piperidine, protected amino acids, 1-hydroxybenzotriazole (HOBt), benzotriazole-1-yloxytris-pyrrolidinophosphonium hexafluorophosphate (PyBOP), and Rink resin which were obtained from Advanced Chemtech and Bachem. Deuterated chloroform ($CDCl_3$) for NMR spectroscopy was purchased from Cambridge Isotope Laboratories. PKCα was purchased from PanVera. Silica gel for flash chromatography (40 mm, 60 Å pore diameter) was purchased from VWR International, and silica gel plates (0.25 mm, UV_{254}) for thin layer chromatography (TLC) were purchased from Analtech.

[00135] The numbering of compounds in Example III is independent of the numbering used in Examples I and II.

[00136] Fluorescence assays were performed using a Photon Technology QM-1 spectrofluorimeter, and irradiation experiments utilized an Oriel Mercury Arc Lamp (Model 69907) equipped with a 360 nm colored glass filter (300-400 nm band pass) and an infrared (IR) filter. ^1H and ^{13}C nuclear magnetic resonance (NMR) spectra were recorded on a Brucker DRX300 (^1H: 300 MHz, ^{13}C: 75 MHz) spectrometer. All NMR chemical shifts (δ) are reported as ppm (parts per million) values and coupling constants (J) in hertz (Hz). ^1H and ^{13}C NMR signals were referenced relative to the chloroform (^1H: 7.24 ppm, ^{13}C: 77.0 ppm) solvent signal. Mass spectra by MALDI (Matrix Assisted Laser Desorption Ionization) were analyzed on an Applied Biosystems Voyager-DE STR mass spectrometer, and mass spectra by ESI (Electrospray Ionization) were analyzed on a Finnigan LCQ mass spectrometer equipped with a quadrupole ion trap. High pressure liquid chromatography (HPLC) analysis was performed using a Rainin Dynamax SD-200 solvent delivery system. Analyses were carried out either on analytical scale (Varian Microsorb-MV C-18, 300 Å particle size, 250 x 4.6 mm) or on preparative scale using three radial compression modules (Waters Delta-Pak C-18, 300 Å particle size, 25 x 10 cm) connected in series.

[00137] *Synthesis of N-(9-Fluorenylmethyloxycarbonyl)-L-serine allyl ester (Compound 3).* N-α-Fmoc-L-Serine (1.64 g, 5.01 mmol) and $NaHCO_3$ (0.43 g, 5.06 mmol) in 16 mL of water were combined with a solution of 2.00 g of tricaprylmethylammonium chloride (aliquot 336) and allyl bromide (3.20 g, 26.5 mmol) in

30 mL of methylene chloride, and the suspension was vigorously stirred at room temperature for 24 hours. Water (50 mL) was added to the reaction mixture, and the suspension was extracted with methylene chloride (3 x 50 mL). The combined organics were dried (Na_2SO_4) and concentrated under reduced pressure, and the crude residue was purified by silica gel chromatography (3:2 hexanes:ethyl acetate) to yield **3** as a white solid (1.70 g, 93%): ^{1}H NMR (300 MHz, $CDCl_3$) δ 2.12 (bs, 1H), 3.92 (d, J = 9.0, 1H), 4.01 (d, J = 9.0, 1H), 4.20 (t, J = 6.8, 1H), 4.41 (m, 3H), 4.67 (d, J = 5.3, 2H), 5.25 (dd, J = 10.4 and 1.0, 1H), 5.32 (dd, J = 17.2 and 1.0, 1H), 5.72 (d, J = 6.1, 1H), 5.88 (ddt, J = 17.2, 10.4 and 5.3, 1H), 7.29 (t, J = 7.4, 2H), 7.39 (t, J = 7.4, 2H), 7.58 (d, J = 7.4, 2H), 7.75 (d, J = 7.4, 2H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 47.1, 56.1, 63.3, 66.4, 67.2, 119.0, 120.0, 125.1, 127.1, 127.7, 131.3, 141.3, 143.6, 143.8, 156.2, 170.1.

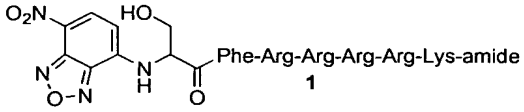
[00138] *Synthesis of 4,5-dimethoxy-2-nitrobenzyl trichloroacetimidate (Compound 4).* 4,5-dimethoxy-2-nitrobenzyl alcohol (1.23 g, 5.77 mmol) was dissolved in 40 mL of anhydrous methylene chloride and stirred at room temperature under nitrogen. Anhydrous K_2CO_3 (2.05 g, 14.8 mmol), trichloroacetonitrile (2.10 g, 10.0 mmol), and anhydrous triethylamine (720 mg, 7.12 mmol) were added to the solution and the reaction mixture stirred at room temperature for 24 hours. Methylene chloride (60 mL) was added to the reaction mixture, and the suspension sequentially washed with 0.5 N HCl and saturated NaCl. The organic layer was dried and concentrated under reduced pressure, and the resulting crude solid recrystallized to yield **4** as an orange powder (1.61 g, 85%): ^{1}H NMR (300 MHz, $CDCl_3$) δ 3.94 (s, 3H), 3.94 (s, 3H), 5.76 (s, 2H), 7.21 (s, 1H), 7.73 (s, 1H), 8.48 (s, 1H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 56.4, 67.5, 91.2, 108.1, 109.2, 127.3, 139.4, 148.1, 153.8, 161.7.

[00139] *Synthesis of N-(9-Fluorenylmethyloxycarbonyl)-O-(4,5-dimethoxy-2-nitrobenzyl)-L-serine allyl ester (Compound 5)* (see Scheme 6 below). Triflic acid (4 μL) was added to an anhydrous methylene chloride (4 μL) solution of Fmoc-serine allyl ester **3** (140 mg, 0.38 mmol) and the acetimidate **4** (530 mg, 1.48 mmol) kept under nitrogen at room temperature. The resulting dark green solution was stirred for 20 min at room temperature. Further addition of triflic acid (4 μL) was performed twice at 20 min intervals. TLC analysis indicated that **4** had disappeared following the final addition of triflic acid. Chloroform (25 mL) and silica gel (~ 2 g) were added to the reaction mixture and then concentrated under reduced pressure. The crude residue, adsorbed on silica, was purified by silica gel chromatography (3:1 hexanes:ethyl acetate) to yield **5** as a light

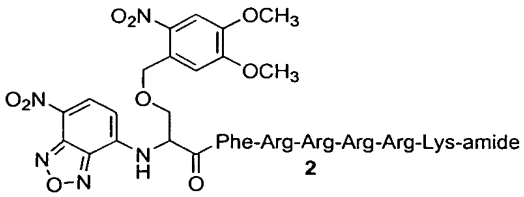
yellow solid (61 mg, 29%): ^{1}H NMR (300 MHz, $CDCl_3$) δ 3.90 (m, 1H), 3.91 (s, 3H), 3.92 (s, 3H), 4.06 (dd, J = 9.3 and 3.0, 1H), 4.21 (t, J = 7.0, 1H), 4.37 (dd, J = 10.5 and 7.0, 1H), 4.45 (dd, J = 10.5 and 7.0, 1H), 4.63 (m, 1H), 4.67 (d, J = 5.3, 2H), 4.91 (AB quartet, J_{AB} = 15.4, 2H), 5.20 (d, J = 10.5, 1H), 5.30 (d, J = 17.2, 1H), 5.69 (d, J = 8.4, 1H), 5.87 (ddt, J = 17.2, 10.5 and 5.3, 1H), 7.14 (s, 1H), 7.28 (t, J = 7.4, 2H), 7.37 (t, J = 7.4, 2H), 7.58 (m, 2H), 7.68 (s, 1H), 7.73 (d, J =7.4, 2H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 47.1, 54.6, 56.3, 56.4, 66.2, 67.1, 70.2, 71.1, 107.9, 109.4, 118.8, 120.0, 125.0, 127.0, 127.7, 129.9, 131.3, 139.0, 141.3, 143.6, 143.8, 147.7, 153.8, 155.9, 169.9.

[00140] *Synthesis of N-(9-Fluorenylmethyloxycarbonyl)-O-(4,5-dimethoxy-2-nitrobenzyl)-L-serine (Compound 6).* The caged serine allyl ester **5** (73 mg, 0.13 mmol) was dissolved in chloroform (3.5 mL) and acetic acid (0.1 mL). N-methylmorpholine (0.4 mL), and $Pd(Ph_3P)_4$ (462 mg, 0.40 mmol) were added to the solution. The reaction mixture was stirred for 4 hours at room temperature, and the reaction was quenched by the addition of 0.1 N HCl (30 mL). The suspension was extracted with ethyl acetate, dried (Na_2SO_4), and concentrated under reduced pressure. The crude residue was purified by silica gel chromatography (39:1 chloroform:methanol) to yield **6** as a light yellow solid: ^{1}H NMR (300 MHz, $CDCl_3$) δ 3.78 (m, 1H), 3.84 (s, 3H), 3.88 (s, 3H), 4.08 (d, J = 7.9, 1H), 4.19 (t, J = 6.7, 1H), 4.38 (dd, J = 10.4 and 6.7, 1H), 4.45 (dd, J = 10.4 and 6.7, 1H), 4.62 (m, 1H), 4.86 (AB quartet, J_{AB} = 15.2, 2H), 5.70 (d, J = 8.3, 1H), 7.04 (s, 1H), 7.27 (t, J = 7.3, 2H), 7.36 (t, J = 7.3, 2H), 7.56 (m, 2H), 7.60 (s, 1H), 7.71 (d, J = 7.3, 2H), 8.25 (bs, 1H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 47.0, 54.3, 56.2, 67.3, 70.2, 70.8, 107.8, 109.2, 120.0, 125.0, 127.1, 127.8, 129.5, 139.0, 141.3, 143.5, 147.6, 153.7, 156.1, 174.9. HRMS (FAB) m/z 523.1721 (M + H^+); Calculated for $C_{27}H_{26}N_2O_9$ (M + H^+): 523.1717.

[00141] *Synthesis of peptides 1 and 2.*



1



2

The peptides were synthesized using a standard Fmoc/benzotriazole-1-yl-oxy-tris-pyrrolidino-phosphonium hexafluorophosphate (PyBOP) peptide synthesis protocol. Each amino acid was attached to the rink amide resin (500 mg, 0.6 mmol/g) via manual

solid phase synthesis according to the following protocol: (a) 1 x 15 mL of 30% piperidine in DMF (30 min); (b) 2 x 15 mL of methylene chloride; (c) 2 x 15 mL of isopropyl alcohol; (d) 2 x 15 mL of DMF; (e) Three equivalents of the Fmoc-protected amino acid, PyBOP, HOBt hydrate, and six equivalents of N-methyl morpholine in 15 mL of DMF (120 min) (f) 2 x 15 mL of methylene chloride; (g) 2 x 15 mL of isopropyl alcohol; (h) 2 x 15 mL of DMF. After the addition of Fmoc-phenylalanine, a 10% aliquot of the resin was removed and the appropriate Fmoc-serine derivative was attached to the resin as described above. After completion of the peptide synthesis, the Fmoc was removed with 3 mL of 30% piperidine in DMF (30 min), and the deprotected peptide treated with 10 equivalents of 4-chloro-7-nitrobenzofurazan (NBD-Cl) and 5 equivalents of diisopropylethylamine (DIEA) in 3 mL of DMF to furnish the resin-linked peptide. The resin was transferred to a 20 mL glass vial, and the peptide was cleaved from the resin using 5 mL of 95:2.5:2.5 trifluoroacetic acid (TFA):triisopropylsilane:H_2O (6 hr). The resin was filtered using a sintered glass filter funnel, and the filtrate was washed with 25% TFA in methylene chloride. The supernatant was concentrated to ~2 mL under a stream of nitrogen, and ethyl ether (25 mL) was added to the remaining supernatant. The resulting suspension was extracted with H_2O (2 x 25 mL), and the aqueous extracts were lyophilized, redissolved in H_2O (30 mL), and purified by preparative reverse-phase HPLC (gradient A: 0.1% TFA in H_2O; solvent B: 0.1% TFA in acetonitrile; 12 mL/min): 0 - 3 min (100% gradient A); a linear gradient from 3 to 40 min (50% gradient A and 50% solvent B); a steep final linear gradient to 100% solvent B for cleaning purposes. After isolation from the HPLC, samples were lyophilized to yield peptides **1** (13 mg, 32% yield) and **2** (12 mg, 34% yield) as a yellow powder. LRMS (ESI) for **1**: m/z 1166.6 (M^+); Calculated for $C_{48}H_{78}N_{24}O_{11}$ (M^+) 1166.6. LRMS (ESI) for **2**: m/z 1361.8 (M^+); Calculated for $C_{57}H_{87}N_{25}O_{15}$ (M^+) 1361.7.

[00142] *Fluorescence PKC assay.* In a 100 μL cuvette, a solution (97 μL) containing irradiated or non-irradiated peptide **2** (60 μM initial concentration), assay buffer, and ATP was allowed to equilibrate at 30 °C for 3 min and the reaction was then initiated via addition of a PKCα stock solution (3 μL). Final conditions were as follows: 61.5 mM HEPES, pH 7.4, 3.0 mM $MgCl_2$, 0.3 mM $CaCl_2$, 0.8 mM dithiothreitol, 0.5 μg/mL phosphatidylserine, 0.1 mg/mL diacylglycerol, 1 mM ATP, 0.1 mM EDTA, 0.1 mM EGTA, 7.5 mM NaCl, 0.002% Triton X-100, 1.5% glycerol, and 43 nM PKC. The assay

solution was gently mixed and continuously monitored for time-dependent change in fluorescent intensity (exitation, 520 nm; emission, 560 nm).

[00143] *Irradiation of the caged peptide for various times intervals and the extent of conversion to uncaged sensor, and the effect on the PKC catalyzed time-dependent change in fluorescence* (Figure 5). The caged peptide **2** (24 μL, 500 μM) was placed in a 1000 μL cuvette and cooled to 0 °C. The sample was irradiated at 150 W for 0, 15, 30, 60, or 90 sec. A 12 μL aliquot was removed and analyzed for change in fluorescence intensity. Another 10 μL aliquot was analyzed by analytical scale reverse-phase HPLC (gradient A: 0.1% TFA in H_2O; solvent B: 0.1% TFA in acetonitrile; 1 mL/min): 0 - 3 min (100% gradient A,); a linear gradient from 3 to 40 min (50% gradient A and 50% solvent B); a steep final linear gradient to 100% solvent B for cleaning purposes. Peptide **2** and its uncaged analogue **1** was quantified by their absorbance at 465 nm and normalized relative to the HPLC peak areas of their pure reference compounds. Average values of three determinations were obtained to calculate the peptide ratios and a standard deviation was calculated to determine the error.

[00144] *Time-dependent change in fluorescence before and after the in situ irradiation of the caged peptide.* The PKC assays were performed as described herein. The caged peptide **2** was incubated at 30 °C with PKCα and ATP, and the change in fluorescence measured for 10, 20, or 30 min. The cuvette was removed from the spectrofluorimeter and irradiated at 150 W for 90 sec. The sample was allowed to re-equilibrate to 30 °C for 1 min and then re-analyzed for change in fluorescence intensity as a function of time.

[00145] *Time-dependent change in fluorescence following irradiation of* **2** *at two different time points.* The PKC assays were performed as described herein. The caged peptide **2** was irradiated at 150 W for 60 sec in the assay buffer containing PKCα and ATP. The sample was then incubated at 30 °C and the change in fluorescence measured for 20 min. The cuvette was then removed from the spectrofluorimeter and again irradiated at 150 W for 60 sec. The sample was allowed to re-equilibrate at 30 °C for 1 min and then re-analyzed for change in fluorescence intensity.

[00146] *Quantum yield determination for the photoconversion of* **2** *to* **1**. A 200 μL solution of 2-nitrobenzaldehyde (300 μM) or **2** (300 μM) in 5 mM HEPES (pH 7.4) was placed in a 1 mL cuvette and irradiated at 150 W for 15 sec or 60 sec, respectively. The product, 2-nitrosobenzoic acid, was quantified by its absorbance at 260 nm and normalized relative to the HPLC peak areas of its precursor (2-nitrobenzaldehyde). The

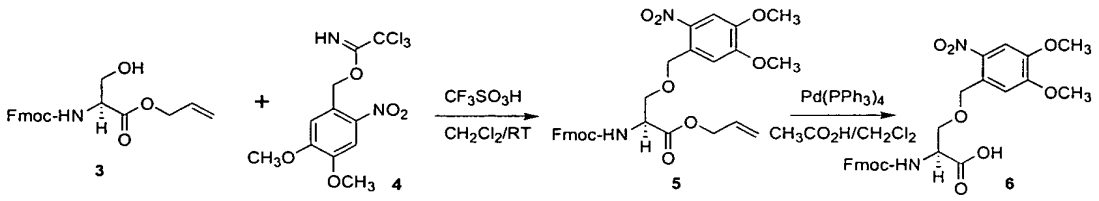
product, **1**, was quantified by its absorbance at 465 nm and normalized relative to the HPLC peak areas of a standard sample of **1**. Average values of three determinations were utilized, and the quantum yield was determined by the following equation: 0.5(%**1**/60 sec)/(%2-nitrosobenzoic acid/15 sec). The quantum yield for the photoconversion of **2** to **1** is 0.059 ± 0.005 based on the reported quantum yield of 0.5 for the photoconversion of 2-nitrobenzaldehyde to 2-nitrosobenzoic acid.[79]

[00147] *Cell-based studies.* HeLa cells were cultured on Lab-Tek II Chamber Slide (single well glass slide) (Nalge Nunc International Corp., Naperville, IL) at 50,000 cells/mL with total volume of 2 mL of serum free Dulbecco's modified Eagle's medium in humidified atmosphere containing 5% CO_2. The caged NBD-containing peptide **2** was dissolved in doubly distilled H_2O at the concentration of 200 μM and was prefiltered through a 0.22 μm filter. Cells were microinjected using a commercial microinjection system (Transjectors 5246, Eppendorf, Westbury, NY) at an estimated final concentration of 20 μM for the caged peptide. Following microinjection, cells were exposed to 365 nm at 1 J/cm^2 to activate the PKC sensor. Immediately after UV treatment, TPA (1 μM) was added into the media to stimulate PKC activity. Time-lapse images were collected with 2 x 2 binning using a Photometrics (Tuscon, AZ) Sensys cooled CCD camera mounted on an Olympus 1 X 70 inverted microscope (Melville, NY) with a PlanApo 40X N. A. 0.75 objective, Ludl shutters (Hawthorne, NY), and a filter set with an excitation wavelength of 460 - 500 nm and an emission wavelength of 510 - 560 nm. Images were collected at 0.5 min, 2 min, 5 min, 10 min, 15 min, 20 min, and 30 min after addition of TPA or phosphate buffered saline (PBS) as negative control (1000 ms exposure time). Images and fluorescence intensity measurements were obtained from both TPA stimulated cells and control cells for photobleaching studies. Images and fluorescence intensity measurements were also collected from caged and uncaged PKC sensors in cells stimulated with TPA.

2. Synthesis of Caged Compound, Results, and Discussion

[00148] Two different strategies were envisioned for the construction of caged protein kinase sensors. PKC is known to recognize peptides containing appropriately positioned arginine residues.[80] Consequently, a substrate harboring multiply caged arginine moieties[11] should be resistant to PKC-catalyzed phosphorylation until activated by light. Alternatively, the presence of a single photolytically sensitive substituent on the

phosphorylatable serine hydroxyl should likewise preclude PKC-catalyzed phosphoryl transfer. Caged thiophosphorylated and phosphorylated threonine and serine residues[81-83] and chain caged serine have been reported;[87-88] however, it is believed that a side chain caged protein kinase fluorescent substrate has not previously been described. The latter offers the advantage, relative to a peptide containing multiply caged residues, that only a single functional group need be photolytically liberated (peptide **2**) to generate the active protein kinase fluorescent reporter. A caged serine was prepared as outlined in Scheme 6. The key step, benzylation of the serine side chain hydroxyl, was achieved using the trichloroacetimidate **4** in the presence of a catalytic amount of triflic acid.[84-85] The Fmoc derivative **6** was subsequently employed to create the active site-directed peptide **2** via solid phase peptide synthesis as described above.



Scheme 6

[00149] As would be expected for a peptide lacking a free hydroxyl group, compound **2** fails to serve as a substrate for PKC (Figure 6). Two potentially useful attributes of the caged substrate include (1) sampling of protein kinase activity at a time of choice and (2) control over the amount of active substrate available for phosphorylation. The former is illustrated in Figure 6, where the caged substrate **2** is incubated with active PKC for various time intervals (10, 20, and 30 min) and then subsequently photoactivated (Hg arc lamp for 90 sec). A broad bandpass filter (300 – 400 nm with λ_{max} @ 360 nm) was employed to protect the nitrobenzofurazan fluorophore from photobleaching and an infrared (IR) filter was used to shield PKC from heat inactivation. Peptide **2** is completely inert as a PKC substrate irrespective of PKC incubation time. Furthermore, identical robust fluorescence responses are immediately observed following photolysis, irrespective of the pre-photolysis PKC incubation time.

[00150] HPLC analysis revealed that the maximal conversion of caged to uncaged substrate is approximately 60%. The quantum yield for photolytic conversion is 0.06 as

determined by actinometry.[79] Although a 90 sec irradiation time is required for maximal *in vitro* formation of the uncaged substrate, intracellular uncaging should proceed more rapidly due to an enhanced photon flux through a comparatively smaller cellular volume.[86] Furthermore, as illustrated in Figure 7, total photon flux can be used to control the amount of free protein kinase probe liberated.

[00151] Both the timing and amount of sensor release can be controlled in a single experiment (Figure 6, insert). Approximately half of compound **2** was photochemically converted to the active probe **1** in the presence of PKC, as indicated by the observed change in fluorescence. Subsequent illumination of the reaction mixture afforded additional free sensor, which likewise furnished a fluorescent response. These experiments demonstrate that it is not only possible to control the timing of protein kinase activity sampling, but that activity measurements can be performed at multiple stages as a function of cellular events. The latter is noteworthy since it establishes that inert sensor can be held in reserve so that kinase activity can be assessed at future time points.

[00152] The light-induced sampling of protein kinase activity was also examined in living cells. The caged protein kinase fluorescent substrate **2** was introduced into HeLa cells via microinjection. Exposure of cells to the phorbol ester TPA activates PKC. Compound **1** serves as a specific sensor for the conventional isoforms of PKC in living cells. However, HeLa cells containing the caged derivative **2** fail to display a fluorescent response upon exposure to TPA alone or upon exposure to light in the absence of TPA. By contrast, a robust response is observed when compound **2**-containing HeLa cells are both illuminated and treated with TPA (Figure 8).

[00153] Prior to the present disclosure, none of the fluorescent reporters described to date allow control over *when* protein kinase activity sampling is performed. The latter property is extremely valuable in a number of instances. For example, cells harboring constitutively active protein kinases can render the intracellular loading of a kinase sensor and the subsequent observation of activity at a well-defined time point problematic. Furthermore, protein kinases can exhibit intermittent activity as a function of some cellular event, such as with PKC, which appears to be activated at several distinct stages during mitosis.[26-30] In general, the ability to control when protein kinase activity is measured with respect to multiple cellular signposts provides the opportunity to collect a large series of parallel temporally-offset samplings of protein kinase action. Disclosed herein is a caged protein kinase sensor prepared by modifying the free hydroxyl group of

a phosphorylatable serine moiety with a photolabile side chain appendage that blocks phosphoryl transfer. It is believed that compound **2** represents the first example of a caged fluorescent reporter of intracellular enzymatic activity. The caged sensor allows one to (1) sample PKC activity with exquisite temporal precision, (2) control the amount of active sensor available for phosphorylation, and (3) examine protein kinase activity at multiple time points.

References

1. Sebolt-Leopold, J. S. (2000) Development of anticancer drugs targeting the MAP kinase pathway. *Oncogene* 19: 6594-6599.
2. Popoli, M., Mori, S., Brunello, N., Perez, J., Gennarelli, M., and Racagni, G. (2001) Serine/threonine kinases as molecular targets of antidepressants: implications for pharmacological treatment and pathophysiology of affective disorders. *Pharmacol. Ther.* 89: 149-170.
3. Agarwal, R. (2000) Cell signaling and regulators of cell cycle as molecular targets for prostate cancer prevention by dietary agents. *Biochem. Pharmacol.* 60: 1051-1059.
4. Senderowicz, A. M. (2001) Development of cyclin-dependent kinase modulators as novel therapeutic approaches for hematological malignancies. *Leukemia* 15: 1-9.
5. Lawrence, D. S., and Niu, J. (1998) Protein kinase inhibitors: the tyrosine-specific protein kinases. *Pharmacol. Ther.* 77: 81-114.
6. Ng, T., Squire, A., Hansra, G., Bornancin, F., Prevostel, C., Hanby, A., Harris, W., Barnes, D., Schmidt, S., Mellor, H., Bastiaens, P. I. H., and Parker, P. J. (1999) Imaging protein kinase Calpha activation in cells. *Science* 283: 2085-2089.
7. Nagai, Y., Miyazaki, M., Aoki, R., Zama, T., Inouye, S., Hirose, K., Iino, M., and Hagiwara, M. (2000) A fluorescent indicator for visualizing cAMP-induced phosphorylation in vivo. *Nat. Biotechnol.* 18: 313-316.
8. Post, P. L., Trybus, K. M., and Taylor, D. L. (1994) A genetically engineered, protein-based optical biosensor of myosin II regulatory light chain phosphorylation. *J. Biol. Chem.* 269, 12880-12887.
9. McIlroy, B. K., Walters, J. D., and Johnson, J. D. (1991) Phosphorylation-dependent binding of a synthetic MARCKS peptide to calmodulin. *Anal. Biochem.*, 195, 148-152.

10. Bowman, B. F., Peterson, J. A., and Stull, J. T. (1992) Pre-steady-state kinetics of the activation of rabbit skeletal muscle myosin light chain kinase by Ca2+/calmodulin. *J. Biol. Chem.* 267 5346-5354.

11. Wood, J., Koszelak, M., Liu, J., and Lawrence, D. S. (1998) *J. Amer. Chem. Soc.* 120, 7145-7146.

12. Marriot, G., and Walker, J. W. (1999) *Trends Plant Sci.* 4, 330-334.

13. Curley, K., and Lawrence, D. S. (1999) Caged regulators of signaling pathways. *Pharmacol. Therap.*, 82, 347-354.

14. Walker, J. W., Gilbbert, S. H., Drummond, R. M., Yamada, M., Sreekumar, R., Carraway, R. E., Ikebe, M., and Fay, F. S. (1998) Signaling pathways underlying eosinophil cell motility revealed by using caged peptides. *Proc. Natl. Acad. Soc. U.S.A.* 95, 1568-1573.

15. Pirrung, M. C., Drabik, S. J., Ahamed, J., and Ali, H. (2000) Caged chemotactic peptides. *Bioconjug. Chem.* 11, 679-681.

16. Tatsu, Y., Shigeri, Y., Ishida, A., Kameshita, I., Fujisawa, H., and Yumoto, N. (1999) Synthesis of caged peptides using caged lysine: application to the synthesis of caged AIP, a highly specific inhibitor of almodulin-dependent protein kinase II. *Bioorg. Med. Chem. Lett.* 9, 1093-1096.

17. Pan, P., and Bayley, H. (1997) Caged cysteine and thiophosphoryl peptides. *FEBS Lett.* 405, 81-85.

18. Kislauski, E. H., Zhu, X.-C., and Singer, R. (1997) beta-Actin messenger RNA localization and protein synthesis augment cell motility. *J. Cell Biol.* 136, 1263-1270.

19. Lee, T. R., Niu, J., and Lawrence, D. S. (1995) The extraordinary active site substrate specificity of pp60c-src. A multiple specificity protein kinase. *J. Biol. Chem.*, 270, 5375-5380.

20. Kwon, Y.-G., Mendelow, M., and Lawrence, D. S. (1994) *J. Biol. Chem.*, 269, 16725-16729.

21. Lee, T. R., Mendelow, M., Srinivasan, J., Kwon, J.-G., and Lawrence, D. S. (1993) *J. Amer. Chem. Soc.* 115, 9888-9891.

22. Yan, X., Lawrence, D. S., Corbin, J. D., and Francis, S. H. (1996) *J. Amer. Chem. Soc.* 118, 11684-11685.

23. Lee, T. R., Niu, J., and Lawrence, D. S. (1994) Phenol kinase activity of the serine/threonine-specific cAMP-dependent protein kinase: steric and electronic effects. *Biochemistry* 33, 4245-4250.

24. Yan, X., Curley, K., and Lawrence, D. S. (2000) The specificity of the protein kinase C alpha, betaII and gamma isoforms as assessed by an unnatural alcohol-appended peptide library. *Biochem. J.* 349, 709-715.

25. Glass, D. B., Masaracchia, R. A., Feramisco, J. R., and Kemp, B. E. (1978) Isolation of phosphorylated peptides and proteins on ion exchange papers. *Anal. Biochem.* 87, 566-575.

26. Goss, V. L., Hocevar, B. A., Thompson, L. J., Stratton, C. A., Burns, D. J., and Fields, A. P. (1994) Identification of nuclear beta II protein kinase C as a mitotic lamin kinase. *J. Biol. Chem.* 269, 19074-19080.

27. Collas, P. (1999) Sequential PKC- and Cdc2-mediated phosphorylation events elicit zebrafish nuclear envelope disassembly. *J. Cell Sci.* 112, 977-987.

28. Passalacqua, M., Patrone, M., Sparatore, B., Pedrazzi, M., Melloni, E., and Pontremoli, S. (1999) Protein kinase C-theta is specifically activated in murine erythroleukaemia cells during mitosis. FEBS Lett. 1999 Jun 25;453(3):249-53. *FEBS Lett.* 453, 249-253.

29. Takai, Y., Ogawara, M., Tomono, Y., Moritih, C., Imajoh-Ohmi, S., Tsutsumi, O., Taketani, Y., and Inagaki, M. (1996) Mitosis-specific phosphorylation of vimentin by protein kinase C coupled with reorganization of intracellular membranes. *J. Cell Biol.* 133, 141-149.

30. Varlamova, O., Spektor, A. and Bresnick, A. R. (2001) Protein kinase C mediates phosphorylation of the regulatory light chain of myosin-II during mitosis. *J. Muscle Res. Cell Motil.* 22, 243-250.

31. Way, K. J., Chou, E., and King, G. L. (2000) Identification of PKC-isoform-specific biological actions using pharmacological approaches. *Trends Pharmacol . Sci.* 21, 181-187.

32. Lingameneni, R., Vysotskaya, N., Duch, D. S., and Hemmings Jr., H. C. (2000) Inhibition of voltage-dependent sodium channels by Ro 31-8220, a 'specific' protein kinase C inhibitor. *FEBS Lett.* 473, 265-268.

33. Davies, S. P., Reddy, H., Caivano, M., and Cohen, P. (1999) *Biochem. J.* 351, 95-105.

34. Gschwendt, M., Kittstein, W., and Marks F. (1991) Protein kinase C activation by phorbol esters: do cysteine-rich regions and pseudosubstrate motifs play a role? Trends *Biochem. Sci.* 16, 167-169.

35. Goekjian, P. G., and Jirousek, M. R. (1999) Protein kinase C in the treatment of disease: signal transduction pathways, inhibitors, and agents in development. *Curr. Med. Chem.* 6, 877-903.

36. Pines, J. Four-dimensional control of the cell cycle. *Nat. Cell Biol.* 1999, 1:E73-9.

37. Blatt, N. B.; Glick, G. D. Signaling pathways and effector mechanisms pre-programmed cell death. *Bioorg. Med. Chem.* 2001, 9, 1371-1384.

38. Lee, C. L.; Linton, J.; Soughayer, J. S.; Sims, C. E.; Allbritton, N. L. Localized measurement of kinase activation in oocytes of Xenopus laevis. *Nat. Biotechnol.* 1999, 17, 759-762.

39. Grynkiewicz, G.; Poenie, M.; Tsien, R. Y. A new generation of Ca2+ indicators with greatly improved fluorescence properties. *J. Biol. Chem.* 1985, 260, 3440-3450.

40. Minta, A.; Kao, J. P. Y.; Tsien, R. Y. Fluorescent indicators for cytosolic calcium based on rhodamine and fluorescein chromophores. *J. Biol. Chem.* 1989, 264, 8171-8178.

41. Tsien, R. Y. Fluorescent probes of cell signaling. *Ann. Rev.Neurosci.* 1989, 12, 227-253.

42. Sun, W.-C., Gee, K. R.; Klaubert, D. H.; Haugland, R. P. *J. Org. Chem.* 1997, 62, 6469-6475.

43. Corey, E. J.; Gras, J.-L.; Ulrich, P. *Tetrahedron Lett.* 1976, 809-813.

44. Lee, T. R.; Lawrence, D. S. Acquisition of high-affinity, SH2-targeted ligands via a spatially focused library. *J Med Chem.* 1999, 42, 784-787.

45. Kwon, YG, Mendelow, M, Srinivasan J, Lee TR, Pluskey S, Salerno A, and Lawrence, DS. The active site substrate specificity of the cAMP-dependent protein kinase. *J. Biol. Chem.* 268: 10713-16, 1993.

46. Lee, TR, Till, JH, Lawrence, DS, Miller WT. Precision substrate targeting of protein kinases v-Abl and c-Src. *J. Biol. Chem.* 270: 27022-26, 1995.

47. Srinivasan, J, Koszelak, M, Kwon, Y-G, Lawrence, DS. The design of peptide-based substrates for the cdc2 protein kinase. *Biochemical J.* 309: 927-31, 1995

48. Wood JS, Yan X, Mendelow M, Corbin JD, Francis, SH, Lawrence, DS. Precision substrate targeting of protein kinases. *J. Biol. Chem.* 271: 174-179, 1996.

49. Nazareth LV and Weigel NL. Activation of the human androgen receptor through a protein kinase A signaling pathway. *J. Biol. Chem.* 271: 19900-7, 1996.

50. Dhingra K, Hortobagyi, GN. Critical evaluation of prognostic factors. *Semin. Oncol.* 23:436-45, 1996.

51. Bramson HN, Thomas N, DeGrado WF, Kaiser ET. *J. Amer. Chem. Soc.* 102: 7156-7, 1980.

52. Wei-Chuan Sun, Kyle R. Gee, Dieter H. Klaubert and Richard P. Haugland; J. Org. Chem. 1997, 62, 6469-6475.

53. Zhou, S.; Cantley, L. C. Recognition and Specificity in Protein Tyrosine Kinase-Mediated Signalling. *Trends Biochem. Sci.* 1995, *20*, 470-475.

54. Lev-Ram, V.; Jiang, T.; Wood, J.; Lawrence, D. S.; Tsien, R. Y. Synergies and Coincidence Requirements Between NO, cGMP, and Ca^{2+} in the Induction of Cerebellar Long-Term Depression. *Neuron* 1997, *18*, 1025-1038.

55. Dostmann, W. R.; Taylor, M. S.; Nickl, C. K.; Brayden, J. E.; Frank, R.; Tegge, W. J. Highly Specific, Membrane-Permeant Peptide Blockers of cGMP-Dependent Protein Kinase Ialpha Inhibit NO-Induced Cerebral Dilation. *Proc Natl Acad Sci U S A* 2000, *97*, 14772-7.

56. Yan, X.; Curley, K.; Lawrence, D. S. The Specificity of the Protein Kinase C Alpha, Beta and Gamma Isoforms as Assessed by an Unnatural Alcohol-Appended Peptide Library. *Biochem. J.* 2000, *349*, 709-715.

57. Kwon, Y. G.; Mendelow, M.; Lawrence, D. S. The Active Site Substrate Specificity of Protein Kinase C. *J. Biol. Chem.* 1994, *269*, 4839-4844.

58. Adams, S. R.; Tsien, R. Y. Controlling Cell Chemistry with Caged Compounds. *Annu. Rev. Physiol.* 1993, *55*, 755-784.

59. McCray, J. A.; Trentham, D. R. Properties and Uses of Photoreactive Caged Compounds. *Annu. Rev. Biophys. Biophys. Chem.* 1989, *18*, 239-270.

60. Blohm, D. H.; Guiseppe-Elie, A. New developments in microarray technology. *Curr Opin Biotech* 2001, *12*, 41-7.

61. Fung, E. T.; Thulasiraman, V.; Weinberger, S. R.; Dalmasso, E. A. Protein biochips for differential profiling. *Curr Opin Biotech* 2001, *12*, 65-9.

62. Hunter, T. Protein kinases and phosphatases: the yin and yang of protein phosphorylation and signaling. *Cell* 80: 225-236, 1995.

63. Hunter, T. A thousand and one protein kinases. *Cell* 50: 823-829, 1987.

64. Ghosh, M.; Ichetovkin, I.; Song, X.; Condeelis, J. S.; Lawrence, D. S. Light-Induced F-Actin Severing Via A Caged Cofilin. *J. Amer. Chem. Soc.* 2002, *124*, 2440-2441.

65. Czernik AJ, Girault J-A, Nairn AC, Chen J, Snyder G, Kebabian J, Greengard P. Production of phosphorylation state-specific antibodies. *Methods Enzymol.* 201: 264-283, 1991.

66. Verveer PJ, Wouters FS, Reynolds AR, Bastiaens PI, Quantitative imaging of lateral ErbB1 receptor signal propagation in the plasma membrane. *Science* 290: 1567-70, 2000.

67. Nerbonne JM, Richard S, Nargeot J, Lester HA. New photoactivatable cyclic nucleotides produce intracellular jumps in cyclic AMP and cyclic GMP concentrations. *Nature* 310: 74-76, 1984.

68. Zhang J, Ma Y, Taylor SS, and Tsien RY. Genetically encoded reporters of protein kinase A activity reveal impact of substrate tethering. *Proc. Nat. Acad. Sci.* 98: 14997-15002, 2001.

69. Ting AY, Kain KH, Klemke RL and Tsien RY. Genetically encoded fluorescent reporters of protein tyrosine kinase activities in living cells. *Proc. Nat. Acad. Sci.* 98: 15003-8, 2001.

70. Higashi H, Sato K, Omori A, Sekiguchi M, Ohtake A, Kudo Y. Imaging of Ca2+/calmodulin-dependent protein kinase II activity in hippocampal neurones. *NeuroReport* 7: 2695-700, 1996.

71. Higashi H, Sato K, Ohtake A, Omori A, Yoshida S, Kudo Y. Imaging of cAMP-dependent protein kinase activity in living neural cells using a novel fluorescent substrate. *FEBS Lett.* 414: 55-60, 1997.

72. Ventura C, Maioli M. Protein kinase C control of gene expression. *Crit. Rev. Eukaryot. Gene Expr.* 11: 243-67, 2001.

73. Formisano P, Beguinot F. The role of protein kinase C isoforms in insulin action. *J. Endocrinol. Invest.* 24: 460-7, 2001.

74. Carter CA. Protein kinase C as a drug target: implications for drug or diet prevention and treatment of cancer. *Curr. Drug Targets* 1: 163-83, 2000.

75. Barry OP and Kazanietz MG. Protein kinase C isozymes, novel phorbol ester receptors and cancer chemotherapy. *Curr. Pharm. Des.* 7: 1725-44, 2001.

76. Swannie HC, Kaye SB. Protein kinase C inhibitors. *Curr. Oncol. Rep.* 4: 37-46, 2002.

77. Lawrence DS. Functional proteomics: large scale analysis of protein kinase activity. *Genome Biol.* 2(2): REVIEWS1007, 2001.

78. Black JD. Protein kinase C-mediated regulation of the cell cycle. *Frontiers in Bioscience* 5: d406-423, 2000.

79. Allen, J. M.; Allen, S. K.; Baertschi, S. W. 2-Nitrobenzaldehyde: a convenient UV-A and UV-B chemical actinometer for drug photostability testing. *J. Pharm. Biomed. Anal.* **2000**, *24*, 167-178.

80. Nishikawa, K.; Toker, A.; Johannes, F. J.; Songyang, Z.; Cantley, L. C. *J. Biol. Chem.* **1997**, *272*, 952-960.

81. Bayley, H.; Pan, P. *FEBS Lett.* **1997**, *405*, 81-85.

82. Zou, K.; Cheley, S.; Givens, R. S.; Bayley, H. *J. Amer. Chem. Soc.* **2002**, *124*, 8220-8229.

83. Rothman D. M.; Vazquez, M. E.; Vogel, E. M.; Imperiali, B. *Org. Lett.* **2002**, *4*, 2856-2868.

84. Iversen, T.; Bundle, D. R. *J. Chem. Soc. Chem. Commun.* **1981**, 1240-1241.

85. Wessel, H.-P.; Iversen, T.; Bundle, D. R. *J. Chem. Soc. Perkin Trans.* **1985**, *1*, 2247-2250.

86. Mitchison, T. J. *J. Cell Biol.* **1989**, *109*, 637-652.

87. Pirrung, M. C.; Nunn, D. S. *Bioorg. Med. Chem. Lett.* **1992**, *2*, 1489-92.

88. Cook, S. N.; Jack, W. E.; Xiong, X.; Danley, L. E.; Ellman, J. A.; Schultz, P. G.; Noren, C. J. *Angew. Chem. Int. Ed. Engl.* **1995**, *34*, 1629-30.

89. Dancey J, Sausville EA. Issues and progress with protein kinase inhibitors for cancer treatment. Nat Rev Drug Discov. **2003** Apr;2(4):296-313.

90. McJilton MA, Van Sikes C, Wescott GG, Wu D, Foreman TL, Gregory CW, Weidner DA, Harris Ford O, Morgan Lasater A, Mohler JL, Terrian DM. Protein kinase C varepsilon interacts with Bax and promotes survival of human prostate cancer cells. Oncogene. **2003** Sep 11;22(39):6014-24.

91. Farrow B, Rychahou P, Murillo C, O'connor KL, Iwamura T, Evers BM. Inhibition of pancreatic cancer cell growth and induction of apoptosis with novel therapies directed against protein kinase A. Surgery. **2003** Aug;134(2):197-205.

92. Stergiopoulos SG, Stratakis CA. Human tumors associated with Carney complex and germline PRKAR1A mutations: a protein kinase A disease! FEBS Lett. **2003** Jul 3;546(1):59-64.